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PERLEY SPAULDING, 1878—1960

J. R. Hansbrough

"On September 19, 1878, the population of the small country hamlet of South Royalton, Vermont, was increased by one. I was present at the event. I have never learned whether the reticence of later years was foreshadowed by silence upon reaching this world or not. Possibly someone neglected to encourage loquacity at that time." Rather typically, Perley Spaulding in later years spoke thus of his modest beginnings and his New England ancestry. He lived a long and productive life that ended as peacefully as it began when, on June 12, 1960, he died in Hartford, Connecticut. During the almost 82 intervening years he compiled a record of service to forestry and forest pathology in North America that will be seldom equalled and rarely excelled.

His early days on a Vermont farm and adjacent wooded hills instilled in him a love of nature and a desire to protect and conserve it. He attended grade and high school in Bethel, Vermont, and in 1900 graduated from the University of Vermont with a Bachelor of Science in Agriculture. While there he studied under Professor L. R. Jones and became acquainted with W. A. Orton, then a graduate student. Association with these two pioneers in plant pathology in this country awakened in him an ambition to devote his life to similar work. After graduation he worked briefly in Maine and Connecticut and in February, 1902, reported for duty at St. Louis, Missouri, as Scientific Aide in the Bureau of Plant Industry, U. S. D. A. His immediate superior was Dr. H. von Schrenk, in charge of the "Mississippi Valley Laboratory," located at the Missouri (Shaw) Botanical Garden. The Director of the Garden was Dr. William Trelease. Under these two men he rapidly developed a proficiency in plant pathology, and in 1902 authored his first technical publication. In the same year he entered Washington University, from which he received his Master of Science in 1903 and his Doctor of Philosophy in 1906.

Except for about a year with the Federal Horticultural Board, in 1913–14, Dr. Spaulding spent his entire official life with the Bureau of Plant Industry. In 1907 he transferred from St. Louis to Washington, D. C., as a member of the new Office of Investigations in Forest Pathology, later the Division of Forest Pathology, under the leadership of Dr. Haven Metcalf. In 1925 he opened a field office of the Division in Amherst, Massachusetts, in cooperation with the Northeastern Forest Experiment Station. In 1932 he moved with the Station to New Haven, Connecticut, where he worked continuously until his retirement in 1948.

During this period that spanned the first half of the 20th century, Dr. Spaulding was an active member of the relatively small group of scientists who successfully worked for recognition of the importance and control of plant diseases in North America. He published about 180 papers—USDA bulletins and articles in technical journals—on the cause, prevention, and control of diseases of forest trees. One of his major contributions was on white pine blister rust, introduced into North America from Europe and first discovered on planted white pines in New York in 1909. He recommended a very effective control program, initiated that year and still in use. He later made valuable contributions to other problems, such as control of diseases in forest nurseries and plantations, prevention of heartrot in balsam fir and northern hardwoods, and reduction of fire hazard through improved slash disposal practices. Perhaps his most telling accomplishment, however, was that he was able to bridge the gap between research and practice—to get research results into use. This was recognized when, in 1948, he received the Superior Service Award of the Department of Agriculture. His citation reads:

"For his unusually productive research and outstanding contributions to the science of forest pathology and its application to forest management practices in the United States."

Dr. Spaulding also exerted a considerable indirect influence on the development of forest pathology in North America through the many younger men who worked with him. Many of these men today hold responsible positions in this profession.

True to his New England heritage, Dr. Spaulding did not really retire in 1948. Far from it. As a

collaborator of the Department of Agriculture he continued his lifetime review and analysis of the forest disease situation throughout the world. In 1956 and 1958 he published two comprehensive Agriculture Handbooks, respectively on the diseases of North American trees planted abroad and on the diseases of foreign forest trees planted in North America. Shortly before his death he completed the manuscript for the third and final volume in the series, an annotated list of the forest tree diseases of the world and of their potential importance if introduced into North America. Quietly and effectively, he worked until he completed the task he had set himself.

Dr. Spaulding was married in 1904 to Anna B. Talbot of East Granby, Connecticut. They had no children. She survives him and maintains a home in the modern log cabin that they built in East Granby for their declining years.

Dr. Spaulding was a life member of the American Phytopathological Society and served as Editor-in-Chief in 1921, 1923, and 1924. He was also a member of the American Association for the Advancement of Science, the Society of American Foresters, the American Forestry Association, and the Vermont, New England, and Washington (D. C.) Botanical Societies. To his associates in these organizations and his fellow forest pathologists he will be remembered for his simplicity, steadfastness, and devotion to duty. To all others his many publications serve as a lasting monument to a life dedicated to public service.

STUDY OF THE GROWTH OF CERATOCYSTIS FAGACEARUM IN OAK WOOD WITH THE USE OF AUTORADIOGRAMS

Charles L. Wilson

Assistant Professor, Department of Plant Pathology, University of Arkansas. The author expresses appreciation to Mr. J. R. Montgomery and Dr. D. A. Brown for assistance.

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SUMMARY

Fungus growth in an inoculated oak tree and inoculated sapwood blocks was traced with autoradiograms and stained sections. Sparse growth of *Ceratocystis fagacearum* was found in the vessels, tracheids, and fibers of boles and branches of *Quercus marilandica* and members of the red oak group. Vessels and tracheids were observed occasionally filled with conidia and mycelia. Most of the fungal growth occurred in parenchyma cells of the medullary rays, uniseriate rays, and xylem parenchyma. Penetration of walls was generally through pits, although direct penetration was also observed. Appressoria were sometimes formed prior to penetration. There was extensive invasion of the middle lamellae. The autoradiograms indicated that sulfur-containing diffusates from fungal hyphae were active on starch grains and the middle lamellae. A semisynthetic medium containing 1.25 millicuries of S^{35} per 50 ml, inhibited growth and sporulation, and was mutagenic. Two mutants, an albino strain and a strain with abundant aerial mycelium, were obtained from transfers of colonies grown on the radioactive medium.

Introduction.—Hyphae and conidia of the oak-wilt fungus, *Ceratocystis fagacearum* (Bretz) Hunt, have been detected in vessels of diseased trees (3, 4, 6, 9, 12, 14). Hyphae have also been observed in xylem parenchyma cells and rays (6, 12). Most reports indicated sparse growth in the vessels, with relatively few vessels involved. In *Quercus ellipsoidalis* E. G. Hill fungal growth was rarely observed in vessels or parenchyma cells at the time of initial leaf wilt, but extensive mycelial growth was found in the vessels at later stages. Hyphae were also found to invade the ray cells, xylem parenchyma, and cambium (12).

Little attention has been given the growth of the fungus in living parenchyma cells. Since the protoplasm of parenchyma cells has staining properties similar to those of fungus mycelium, it is difficult to differentiate fungus growth within the cell. This paper is concerned with the development of *C. fagacearum* in oak wood with particular attention being given the growth of the fungus in parenchyma cells and the middle lamella. Both stained sections and autoradiograms were used for study.

Materials and methods.—Material for sectioning was obtained from: 1) the bole and branches of naturally infected blackjack oak (*Quercus marilandica* Muench) and members of the red oak group, all about half defoliated; 2) the bole of an artificially inoculated blackjack oak; 3) blocks of sapwood taken from the bole of blackjack oak and inoculated with *C. fagacearum*. Where naturally infected wood was used, pure cultures of *C. fagacearum* were obtained near the area that was sectioned. Material was fixed in formalin-aceto-alcohol (FAA) and sectioned at 6–12 μ on a sliding microtome. A picro-analine blue and safranin stain (10) was used for differentiating the fungus in the wood.

For the autoradiographic studies, *C. fagacearum* was grown on agar media in Petri dishes with 1.25, 0.50, and 0.25 millicuries of S^{35} P-1 added to 50 ml

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of a basal medium. The basal medium, formulated to contain a relatively low amount of sulfur, consisted of: "Difco" yeast extract (2.0 g), dextrin (10.0 g), asparagine (2.0 g), KH_2PO_4 (1 g), $MgSO_4.7H_2O$ (0.1 g), Fe^{+++} (0.2 mg), Zn^{++} (0.2 mg), Mn^{++} (0.1 mg), water (1.0 l), agar (20.0 g). The isotope was incorporated into the medium before it was autoclaved. Radioactive sulfur was chosen because it would be incorporated partially into cellular protein and thus be retained in the mycelium.

Cultures used for inoculating the standing tree were grown on the radioactive medium for 27 days. Small cuts were made with an ax just into the sapwood of the bole of a blackjack oak, and blocks of radioactive fungal growth were placed on the cut surfaces. Inoculations were made on April 1, 1960, and on April 12, 1960, blocks for sectioning were removed from the area of inoculation. These blocks were fixed for 12 hours in FAA, and sectioned, and autoradiograms were made of the sections.

Small blocks of sapwood ($1 \times 1 \times 2$ in.) were cut aseptically from a living blackjack oak and dipped in paraffin. *C. fagacearum* was grown on the radioactive medium for 4 days, after which small blocks (0.5 sq in.) of fungal growth and subtending agar were removed. The paraffin was removed from one end of the sapwood blocks, and the fungus growth was placed in contact with the exposed surface. This material was placed in sterilized Petri dishes and incubated 7 days at 25°C. The blocks were then fixed in FAA for 12 hours. Sections were cut on a sliding microtome, and affixed to microscope slides with Haupt's adhesive (8).

Autoradiograms were then made by the stripping-film method (5). Kodak AR 10 autoradiographic stripping plates were used. The emulsions were wrapped around the wood sections, allowed to dry, and placed in light-tight boxes, which were stored in a light-tight drawer. The emulsions were exposed for periods of 4–23 days. The film was developed for 5 minutes in Kodak formula D 19b. Good autoradiograms were produced at all the exposure times tested for the sapwood blocks, but a 10-day exposure was necessary to produce interpretable results with wood from the inoculated tree. Results were best with wood inoculated with fungus colonies grown on a medium containing 1.25 millicuries of S^{35} per 50 ml of the basal medium.

If the autoradiograms were allowed to dry completely before a mounting medium and cover slip were placed over them, air bubbles that interfered with observations developed in the wood cells. This problem was overcome by mounting the film and section in lactophenol while the wood cells still contained water and the film had not completely dried. It was possible to stain the sections before enveloping them with the emulsion. Some of the stain diffused into the mounting medium after the autoradiograms were developed, but observations could still be made. Attempts to stain the wood sections after the emulsion was developed were unsuccessful because the film stained so deeply that the section could not be seen. Good alignment between the film and the section was maintained throughout the process of making the autoradiograms.

Results.—*Growth in vessels and tracheids.*—Fungal growth was sparse in the vessels and tracheids of the bole and branches of naturally infected trees. Occasional vessels and tracheids were found that were filled with conidia and hyphae (Fig. 1-A). The conidia were smaller than those normally produced in culture, averaging $1.2 \times 3.5\ \mu$. Many were typical in shape, but some varied from oval to fusiform. Short pieces of hyphae were present in some vessels and tracheids (Fig. 1-B). These hyphal segments apparently produce spores, as sporelike bodies could be seen within some of their walls.

Conidiophores were observed in the half-bordered pits between parenchyma cells and vessels. They originated in the parenchyma cells and extended through the pits into the lumen of the vessels. Conidia could be seen within their walls, and some were observed being liberated into the vessels.

Growth in parenchyma cells and middle lamella.—Hyphae could often be observed entering parenchyma cells, but their activity could not be followed within the cell (Fig. 1-D). The growth of *C. fagacearum* in wood from the inoculated tree and sapwood blocks could be detected with autoradiograms. The exposure on the film and the wood sections were close enough together that both could be viewed simultaneously up to ×712 magnification. The hyphae subtending the exposure on the emulsion could be identified occasionally. The pattern of the exposure on the emulsion generally indicated that the fungus had taken up and emitted the radiation. There was some general diffusion of the S^{35}, which was indicated by a random distribution of dots on the photographic emulsion. However, throughout most of the wood the radiation was localized in individual cells or filamentous lines (Fig. 1-F). In the sapwood blocks, most of the protoplasts and plasma membranes of the parenchyma cells remained intact and were not discolored except in areas of hyphal invasion. The results obtained from the sapwood blocks and the inoculated tree were similar and no distinction is made between the two in the presented results.

Most of the growth of the fungus as indicated by the radiation was confined to the parenchyma cells of the medullary rays, uniseriate rays, and xylem parenchyma. Growth within the parenchyma cells was generally next to the wall near the plasma membrane (Fig. 1-E, 2-D). Sometimes individual, isolated cells produced a heavy exposure on the film (Fig. 1-E). In such cases a denser area, resembling hyphae, could often be found. Some cells were traversed by filamentous lines corresponding to hyphae in size and habit (Fig. 1-F, 2-D).

Areas of the middle lamellae of diseased trees often stain blue with picro-analine blue and safranin (10), indicating a change in consistency or the presence

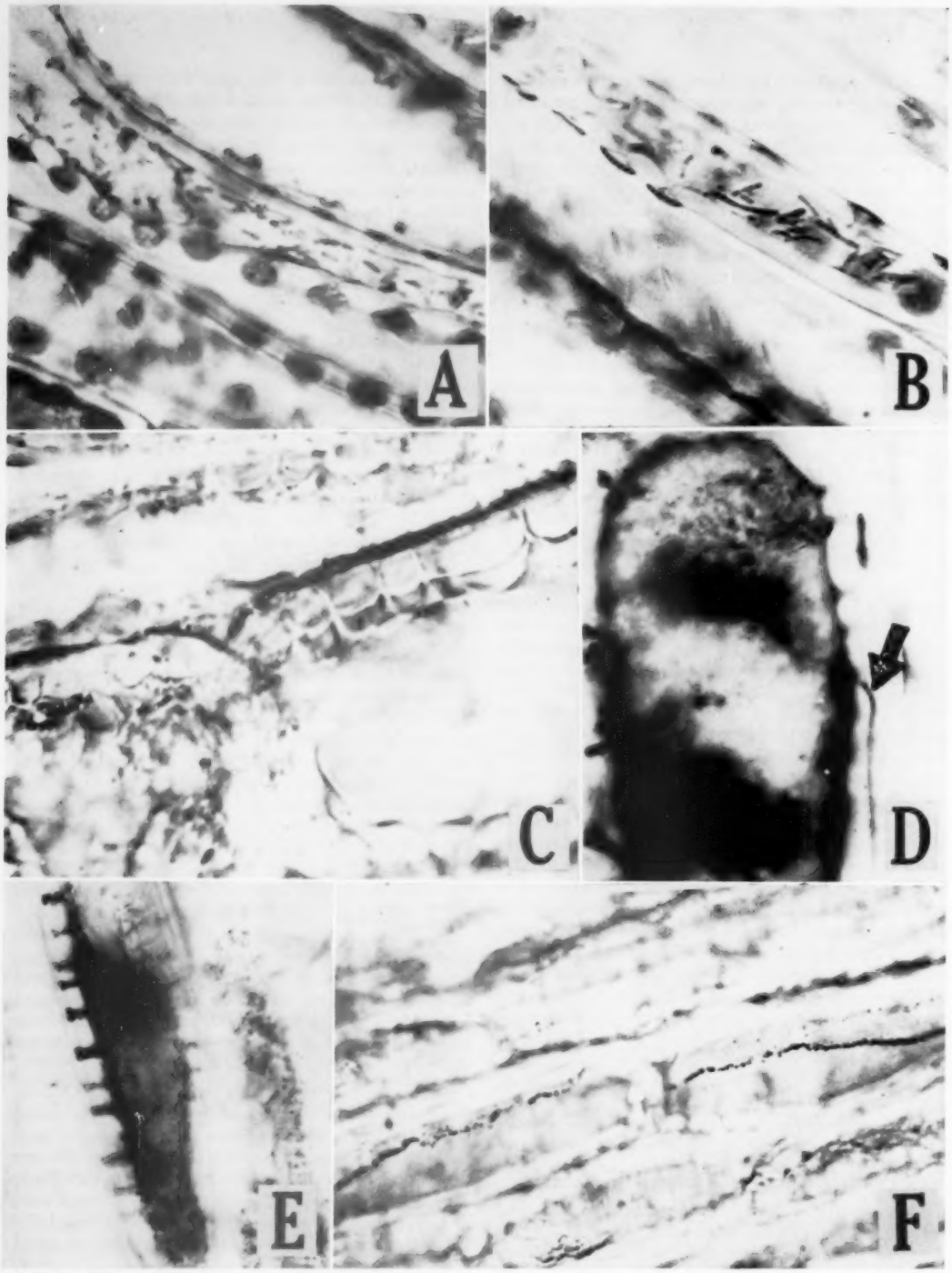

Fig. 1.—Photomicrographs of *C. fagacearum* in oak wood. **A)** Stained section of vessels in naturally infected tree. Note numerous conidia in one vessel (about ×1000). **B)** Same as A, with one vessel filled with hyphal fragments and conidia (about ×1000). **C)** Unstained section and autoradiogram of a portion of 3 parenchyma cells from inoculated tree showing growth in the middle lamella. Dark line in middle lamella is exposure on photographic emulsion (about ×2500). **D)** Stained section of xylem parenchyma cell of inoculated tree. Note stained fungal growth in middle lamella entering lumen of cell (arrow) (about ×1750). **E)** Unstained section and autoradiogram of parenchyma cell in medullary ray of sapwood block. Note dense radiation confined to cell, with a denser area of radiation against wall at left (about ×1400). **F)** Unstained section and autoradiogram of xylem parenchyma cells of inoculated tree. Note filament of radiation passing through 2 cells at center (about ×1400).

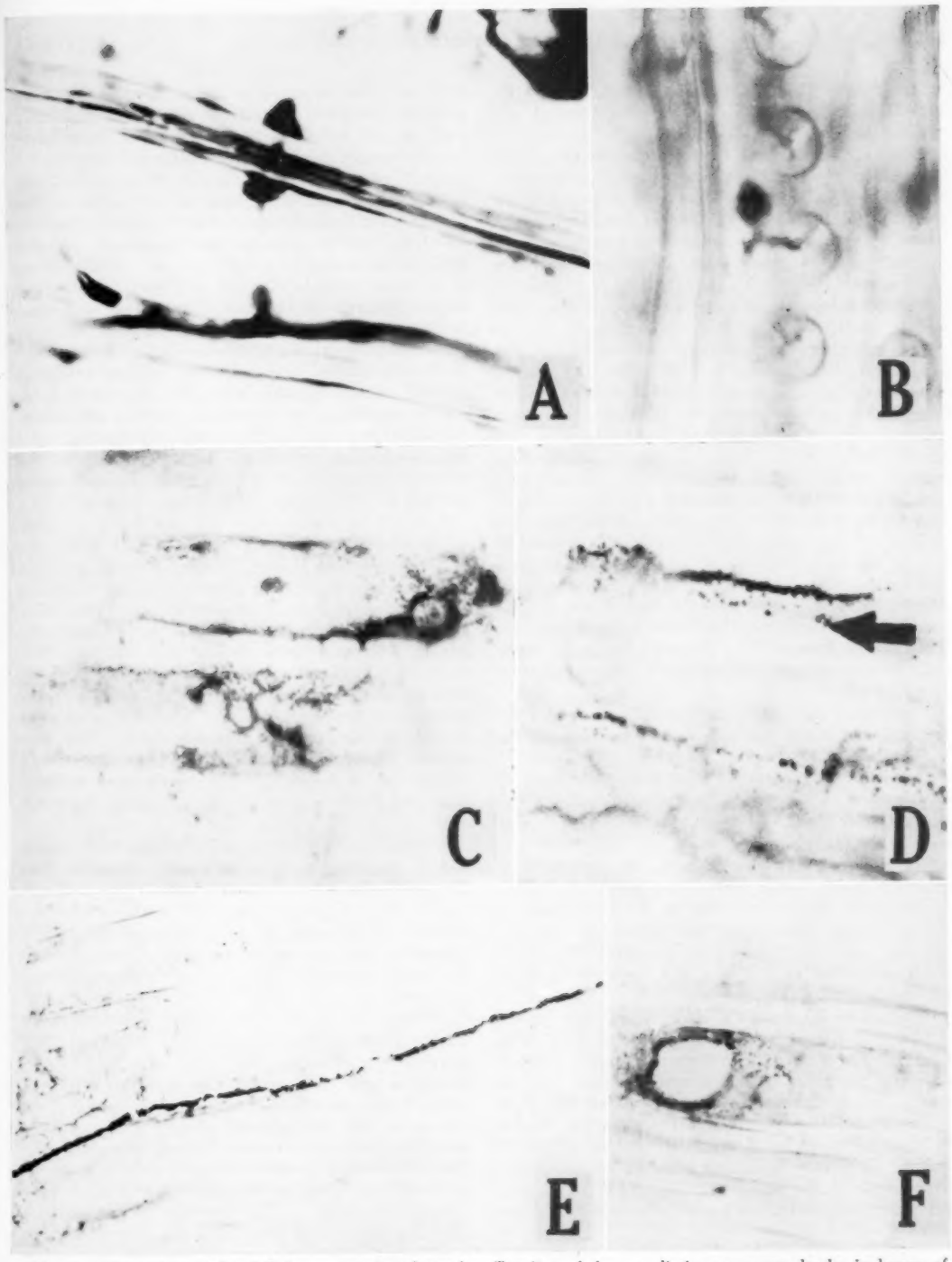

Fig. 2.—Photomicrographs of *C. fagacearum* in oak wood. **A)** Stained section of hyphal growth in vessels of inoculated sapwood block. Note penetration of vessel wall and appressorial formation (about ×1750). **B)** Stained section of vessels of naturally infected tree. Note appressorial formation (about ×2000). **C)** Unstained section and autoradiogram of uniseriate ray parenchyma in inoculated sapwood block. Note concentration of radiation around starch grains (about ×1400). **D)** Unstained section and autoradiogram of uniseriate ray parenchyma in inoculated sapwood block. Top line of dense radiation represents hypha in lumen of cell. Note diffusion of sulfur into middle lamella (arrow). Bottom line of radiation represents hyphal growth in middle lamella (about ×1600). **E)** Unstained section and autoradiogram of xylem parenchyma and vessels. Filamentous line represents growth in middle lamella (about ×1400). **F)** Unstained section and autoradiogram of xylem parenchyma cell. Note concentration of radiation around vacuole (about ×1000).

of the fungus (Fig. 1-D). The autoradiograms revealed considerable radiation in the area of the middle lamella (Fig. 1-C, 2-D,E). The middle lamella in some areas between parenchyma cells, between vessels, and between vessels and parenchyma cells emitted radiation. A filament of dense radiation could be followed from the middle lamella into the lumen of certain parenchyma cells or vessels, indicating that the fungus was present. In the inoculated tree conidia were evident between parenchyma cells in the intercellular space created by the invasion of the middle lamella.

The radiation was generally confined within the fungus mycelium except in cells where the plasma membrane had been destroyed and there was evident digestion of cellular contents. In such cells dense areas of radiation could often be found associated with starch grains (Fig. 2-C). Since starch-digesting enzymes contain sulfur, the area of dense radiation may be one of high enzymatic activity. When a dense filament of radiation could be found adjacent to the wall, a parallel line of radiation could sometimes be seen in the middle lamella (Fig. 2-D). The radiation in the middle lamella in such cases could be associated with pectinase diffusates from the fungal hyphae. The production of pectinases has been demonstrated for the oak-wilt fungus (7). Vacuoles in parenchyma cells were sometimes surrounded by dense radiation (Fig. 2-F).

Penetration and invasion.—Hyphal growth was observed in the xylem parenchyma, uniseriate ray cells, and medullary ray cells. The parenchyma cells of the medullary rays were the most extensively invaded. Hyphae were in the vessels, tracheids, libriform fibers, and tracheid fibers. Of these, the vessels contained most of the fungal growth.

The diameter of the hyphae in the wood varied greatly. Within some parenchyma cells and vessels hyphae were observed as large as 4.0 μ in diameter. Some hyphae, particularly in the middle lamella, were as small as 0.5 μ in diameter (Fig. 2-E). This small threadlike growth was also observed in some parenchyma cells (Fig. 1-F).

Penetration of vessel, tracheid, fiber, and parenchyma cell walls was generally through pits (Fig. 2-B), but direct penetration occurred. Direct penetration was prevalent in the sapwood blocks, and instances were observed in the inoculated tree and naturally occuring oak-wilt trees. Appressoria similar to those formed by *Ceratocystis piceae* (Münch) Bakshi (13) were formed at some points where the hypha entered the wall (Fig. 2-A, B). An oblique penetration of the wall of some parenchyma cells was noted.

Hyphae passed considerable distances through the middle lamellae between parenchyma cells (Fig. 2-E). In some cases the middle lamella of a uniseriate ray was completely invaded by the fungus. Each parenchyma cell was encircled by mycelium.

Some vessels in naturally infected trees were filled with conidia and mycelium. Relatively few vessels were involved in any particular section, however. Invasion was more extensive in the parenchyma cells than in the vessels in trees examined soon after inoculation and trees that were 50% defoliated.

Effect of radiation on fungus in culture.—There was a pronounced effect from the radiation on the growth of *C. fagacearum*. An inhibition of aerial mycelium and sporulation was prominent on the medium containing 1.25 millicuries of S^{35} per 50 ml of the basal medium. A slight inhibition was observed on the medium containing 0.50 millicuries and none on the medium containing 0.25 millicuries. *C. fagacearum* colonies normally develop matlike areas where the perithecia are produced when cultures are spermatized. These matlike areas did not develop on colonies receiving the greatest radiation, and aerial mycelium and sporulation were only sparse. The radiation did not reduce the radial extension of the colonies. The effect of the radiation appeared to be greatest in areas where the agar was thickest.

Transfers were made from colonies that had grown on the radioactive medium containing 1.25 millicuries of S^{35} for 14 days. Three colonies of the albino type and 3 colonies producing abundant aerial mycelium were obtained besides a number of normal colonies. Transfers from colonies grown on the medium containing 0.50 millicuries yielded two cultures that produced abundant aerial mycelium, with the remainder being normal. All transfers from cultures grown on a medium containing 0.25 millicuries were normal in their colony characteristics. The two variants obtained were similar to those obtained in culture and in nature (1, 2). Single-spore cultures from the variants retained their colony characteristics.

Discussion.—Parenchyma cells contain more elaborated compounds essential in fungus nutrition than the vessel fluids and vessel walls. The oak-wilt fungus undergoes considerable growth and reproduction, as indicated by its wide-spread occurrence throughout the sapwood of an affected tree. It does not seem reasonable that it could obtain enough nutriment from the vessels to produce this growth. Its occurrence in the dead vessels would not constitute a parasitic relationship unless certain exogenous enzymes were produced that acted on a substrate in the living cells. This study has revealed that most of the fungus growth of *C. fagacearum* in a wilting tree is in the parenchyma cells where a definite parasitic relationship exists. The occurrence of the fungus in vessels appears to result primarily in the distribution of conidia throughout the tree.

The relationship of the invasion of parenchyma cells and the middle lamella to the wilt mechanism is not clear. The living cells of the rays and xylem parenchyma may be important in water transport. The parenchyma cells in oak form an interconnected framework throughout the sapwood. The invasion of parenchyma cells may induce tylose and gum formation (11). The middle lamella may be important in water transport, and its invasion may be a

factor in the wilt mechanism. Although these are possible explanations, the precise nature of the wilt mechanism remains to be determined.

Chemotherapeutants aimed at the destruction of the fungus in affected trees have been tested with little success. The presence of the fungus primarily in parenchyma cells may partially explain these failures. Systemic materials are needed that will readily diffuse into parenchyma cells and kill the fungus without harming the host cell.

The autoradiographic technique used should prove useful in investigations of other types of parasitism. It provides an opportunity to follow fungal growth and the activity of certain fungal metabolites within living host cells. The mutagenic properties of the radioactive medium may be useful in genetic studies and in the selection of sporulating strains of an isolate. —Department of Plant Pathology, University of Arkansas, Fayetteville.

LITERATURE CITED

1. Barnett, H. L. 1953. A unisexual male culture of Chalara quercina. Mycologia 45:450-457.
2. Barnett, H. L. 1955. Variation in the oak wilt fungus, Endoconidiophora fagacearum. Proc. West Va. Acad. Sci. 27:25-29.
3. Bart, G. J., and H. C. Young. 1958. White oak resists wilt fungus. Ohio Farm and Home Research 43(314): 69-70.
4. Beckman, C. H., J. E. Kuntz, A. J. Riker, and J. G. Berbee. 1953. Host responses associated with the development of oak wilt. Phytopathology 43:448-454.
5. Comar, C. L. 1955. Radioisotopes in biology and agriculture. McGraw-Hill, New York. 481 p.
6. Fergus, C. L., and D. C. Wharton. 1957. Oak wilt: histological studies of host reaction and pathogen. Penn. State Univ. Agr. Expt. Sta. Progr. Rept. 168, 6 p.
7. Fergus, C. L., and D. C. Wharton. 1957. Production of pectinase and growth-promoting substances by Ceratocystis fagacearum. Phytopathology 47:635-636.
8. Johansen, D. A. 1940. Plant microtechnique. McGraw-Hill, New York. 523 p.
9. Parmeter, J. R., Jr., J. E. Kuntz, and A. J. Riker. 1956. Oak wilt development in bur oak. Phytopathology 46:423-436.
10. Proctor, P., Jr. 1941. Penetration of the walls of wood cells by the hyphae of wood-destroying fungi. Yale Univ. School Forestry Bull. 47.
11. Struckmeyer, B. Esther, C. H. Beckman, J. E. Kuntz, and A. J. Riker. 1954. Plugging of vessels by tyloses and gums in wilting oaks. Phytopathology 44:148-153.
12. Struckmeyer, B. Esther, J. E. Kuntz, and A. J. Riker. 1958. Histology of certain oaks infected with the oak wilt fungus, Phytopathology 48:556-561.
13. Wilson, C. L. 1959. Penetration and invasion of Ceratocystis piceae in white oak wood. Mycologia 51: 311-317.
14. Young, R. A. 1949. Studies on oak wilt, caused by Chalara quercina. Phytopathology 39:425-441.

CELL WALL COMPOSITION OF LEAVES WITH A LOCALIZED VIRUS INFECTION

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SUMMARY

Pectinase acting on strips of leaves of *Nicotiana glutinosa* and *N. tabacum* (Connecticut Havana 423) releases twice as many single cells from healthy tissue as from tissue infected locally with TMV. The walls of cells surrounding the lesions, in a radius of about 50 cells, contain predominantly calcium pectate, in contrast to the healthy tissues, which contain primarily pectic acid interspersed with calcium pectate. *In vitro* tests with pectinase and pectic substances indicate that the enzyme hydrolyzes pectic acid more rapidly than calcium pectate, which explains the slower rate of disintegration of infected tissues. Tissues surrounding old lesions on detached leaves contain soluble pectin. The staining reactions of the cells in the lesions indicate some abnormality in the cellulose composition of the walls, while only negative reactions are obtained for pectic substances and for phenolic compounds.

Introduction.—During studies on the metabolic effects of localized virus infections (8,9), single-cell preparations were made from leaves of *Nicotiana glutinosa* and from *N. tabacum* Connecticut Havana 423 (referred to as Conn. 423), both healthy and infected with tobacco mosaic virus (TMV). TMV produces local necrotic lesions on both of these hosts. The method for preparing single cells was a modification of that described by Zaitlin (10), using pectinase to disintegrate the tissues. Details of the metabolic study with these preparations will be published elsewhere.

It was observed, however, that each time a preparation of single cells was made, the number of cells obtained was invariably greater from healthy tissues than from infected tissues in the same period. Since the tissues from infected leaves did eventually disintegrate totally, except for the lesions themselves, we assumed that factors connected with the pectic constitution of the cell wall slowed the rate at which the pectinase released single cells from the intact tissues. The examination of these factors is described here.

Materials and methods.—The single-cell preparations were made by immersing 10–20 g of leaf tissue, sliced into very thin, short strips, in 100 ml of

solution of pectinase (usually 300 mg) in 0.01*M* tris buffer, pH 6.9, which contained sufficient sucrose to make a 0.35*M* solution. Quantities of pectinase (Nutritional Biochemicals Corp.) as low as 100 mg/100 ml and as high as 600 mg/100 ml of solution were used several times. These acted equally well, but respectively more slowly and more quickly. Although Zaitlin (10) observed no pectinase activity at pH 7.0, our results evidenced no marked differences in the disintegrating activity of the enzyme at pH 5.5, 6.9, and 7.2.

The strips of TMV-infected tissues were cut from leaves of *N. glutinosa* and Conn. 423 that had been inoculated with TMV solution concentrated enough to produce a mass of lesions on the entire surface of the leaves. The suspension of leaf strips in the pectinase solution was shaken for 2–3 hours at room temperature on a wrist-action shaker. The cells were then filtered through cheesecloth and gently packed and washed by resuspension in successive slow-speed centrifugations through solutions of 0.01*M* tris buffer with decreasing concentrations of sucrose, until both the pectinase and the sucrose were eliminated.

The single cells were examined with a microscope and appeared to be organizationally intact, displaying considerable Brownian movement in the protoplast. Each preparation was sampled, and the number of cells was estimated with a Howard Mould Counting Chamber. The number of cells from healthy tissues of Conn. 423 was 2–3 million cells/ml, whereas the number of cells from TMV-infected tissues was 1–1.5 million /ml, rarely reaching 2 million /ml. The ratio of the number of healthy to diseased cells was therefore about 2:1. Results were similar with *N. glutinosa*, the ratio of the number of healthy to diseased cells being 2 or 2.5:1.

Since the pectinase acted as a disintegrating agent on the intact tissues, presumably through hydrolysis of the pectic materials in the middle lamella, an examination was therefore made of the pectic contents of healthy and diseased tissues.

The methods used for identifying the pectic materials in the cell walls were those generally used and described previously (7). For the sake of convenience, most of the leaves were removed from the plants and placed in Petri dishes on moist filter paper, after which the lesions developed. Tests made with leaves still attached to the plant were sufficient to ensure that both sources of material gave the same results.

Free-hand sections were cut from small pieces of young *N. glutinosa* leaves, both healthy and infected with TMV for various periods. The sections were of varying thickness, ranging from 1/4 mm to a few cells thick, and those from the infected leaves contained a portion of one or more lesions. Pectic substances were indicated by ruthenium red, which stains these substances an intense pink or cherry-red.

Results.—*Tests for pectic substances.*—Sections of healthy tissues showed the presence of pectic substances in the walls of all cells. In the infected tissues the pectic materials were stained in all areas of the section except in the lesions themselves. The cells in the lesions remained yellow or brown, and the cell walls were unstained except for those of the epidermal and sometimes the first sub-epidermal layers, both of which took up the stain markedly.

Since pectin (the methyl ester of pectic acid) is soluble in hot water, healthy and diseased sections were placed in water, heated 10 minutes at 90°C, and then stained. The distribution of pectic substances was not changed from that in unheated sections. It appeared that there was little or no pectin in the cell walls, or, if present, was of little importance in maintaining tissue integrity.

Since pectic acid forms a soluble potassium salt with KOH, other sections were then immersed in 2% KOH and heated gently for 20 minutes. Within a short time, the thinner areas of healthy sections disintegrated. When the heating was completed, all of the sections of healthy tissue were broken up into very small bits of cellular material. When these pieces were stained with ruthenium red, pectic substances proved to be present only at irregular intervals along the cell wall. These results indicated that the main pectic constituent of the healthy tissues was pectic acid, and that the residue that stained after treatment and held the small pieces together was calcium pectate, which is insoluble in KOH.

Sections from infected tissues, on the other hand, remained intact to a much greater degree under similar treatment with 2% KOH. There was some disintegration, but relatively large pieces of tissue could be taken out of the KOH and examined. These showed pectic substances very strongly on each side of the lesions for 40–60 cells along the length of the section. Beyond this, the staining pattern was much like that observed in the treated healthy tissues. The cells in the lesion itself did not stain at all. These results indicate that within a radius of about 50 cells from the lesion, the pectic substances in the cell wall are not pectic acid interspersed with calcium pectate, but rather, calcium pectate alone.

Calcium pectate can be broken down and pectic acid set free by treatment with HCl. Accordingly, sections of infected tissues were heated gently in 2% HCl, washed, and transferred to 2% KOH. There they disintegrated rapidly and almost completely. Thus, at least in the vicinity of the lesions, pectic acid appears to be converted to calcium pectate. To determine how far this change actually extends, sections were cut from areas successively farther from the periphery of a lesion. Sections from areas more than about 5 mm from the border of a lesion produced solubility reactions the same as in healthy tissues, the pectic substances being primarily pectic acid, with some calcium pectate.

If the leaves were allowed to remain in the Petri dishes at 23–27°C, within a day or two after optimum development of the lesions a yellow area spread outward from the lesions, surrounding them in a

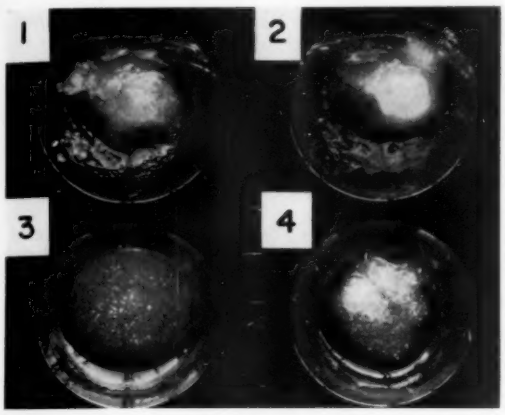

Fig. 1.—The effect of pectinase on calcium pectate and pectic acid, after 5 hours. **1)** 0.2 ml pectinase + 40 mg calcium pectate. Note that the pectate is almost entirely undigested. **2)** 0.2 ml pectinase, inactivated by boiling, + 40 mg calcium petcate. Note lack of digestion. **3)** 0.2 ml pectinase + 40 mg pectic acid. Note that the pectic acid is for the most part digested and in solution. **4)** 0.2 ml inactivated pectinase + 40 mg pectic acid. The swelling occurred immediately upon addition of the enzyme, but digestion has not proceeded further.

roughly circular fashion. The healthy control leaves also became yellow, but in a much more even way throughout the whole leaf blade, and this process began about 2 days after chlorosis was marked around the lesions. When sections were cut from the chlorotic areas around these older lesions and stained with ruthenium red, a distribution of pectic substances similar to that in healthy tissues was observed in the cell walls, but not in the lesions themselves. When such sections were heated 10 minutes in water at 90°C they disintegrated completely, not leaving fragments large enough for microscopic examination. This indicated that the areas surrounding the old lesions now contained water-soluble pectin in the middle lamella.

In vitro tests with pectinase.—Since calcium pectate has been shown to produce greater firmness in cell walls than does pectic acid (3), it was possible that the resistance of the TMV-infected tissues to disintegration by pectinase was due to the properties of this substance, which had been shown to be present around the many lesions on these leaves. An attempt was therefore made to obtain at least a qualitative estimate of the relative rates of hydrolysis of pectic acid and calcium pectate by pectinase.

Accordingly, both pectic acid and calcium pectate were prepared from pectin by methods described by Joslyn (4). The pectic acid, after the final drying, yielded a preparation of small, hard lumps, whereas the calcium pectate formed a thin, translucent sheet. Forty mg of each of these substances was ground into a dry paste with 0.1 ml water, and heaped in the center of wells of a porcelain plate. To each was added 0.2 ml of pectinase (10 mg/ml) in tris buffer, 0.01*M*, pH 6.9. As controls, 0.2 ml of the pectinase solution inactivated by boiling for 3 minutes, was added to another 40 mg of each substance.

Immediately on the addition of the enzyme solutions, both of the pectic materials took up the liquid and swelled to some extent, the pectic acid more so than the calcium pectate. After 5–9 hours, the pectic acid in the active enzyme was completely digested and was in solution, whereas the calcium pectate had undergone no further change (Fig. 1). Neither of the samples in the boiled enzyme changed. Twenty-four hours later the calcium pectate had begun to digest and dissolve a slight amount, but was still mostly intact. It was not until 36 hours that the lump of calcium pectate was clearly diminished and in the process of solution. It appeared, therefore, that pectinase hydrolyzes pectic acid much more readily than it hydrolyzes calcium pectate.

Tests with the lesions.—When the strips of TMV-infected tissue were shaken in the pectinase solution for 9–15 hours, all the tissues outside of the necrotic lesions disintegrated, leaving only the isolated lesions (Fig. 2). This raised a question as to why the lesions should be impervious to pectinase digestion. The lesions did not show the presence of pectic substances by staining with ruthenium red, so that several possible explanations offered themselves. Either there were none of the usual pectic substrates in the cell walls of the lesioned areas, or some substance masked the normal staining reactions, or the lesions were impermeable to aqueous solutions such as that of ruthenium red and pectinase.

To test these possibilities, other stain reactions were attempted. Methylene blue stains pectic substances violet, and other membrane substances blue or green. When methylene blue was applied to sections with lesions, all areas including the parenchyma cells, vascular elements whenever present, and epidermal cells took up the dye readily in the cell walls, producing a deep blue or blue-violet in the middle

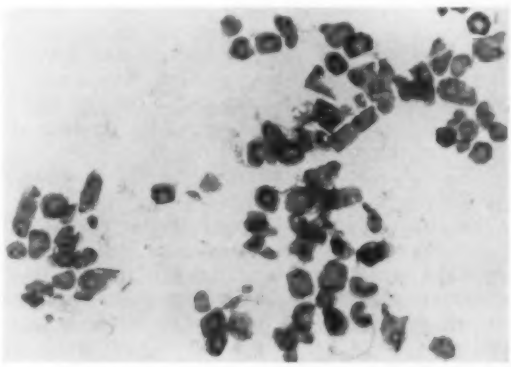

Fig. 2.—Isolated local necrotic lesions from *N. glutinosa* infected with TMV. These were recovered from the suspension of leaf strips in pectinase solution after 12 hours of shaking (× 1.5).

lamellae, and blue in the cell walls generally. Other parts of the cells, except for some nuclei, stained very lightly or not at all. On the other hand, the cells composing the lesions showed no stain at all in the cell walls, or at most a light green, whereas the cell contents took up the dye strongly, producing a very dark blue. (This stain also showed clearly that the shape of the lesion cells was no longer rectangular in the palisade area, but was rounded throughout the whole area of the lesion.) It appeared, then, that an aqueous solution, in this case of methylene blue, could reach the interior of the lesion but still not show the presence of pectic substances.

The cellulose portion of the cell walls in the lesions was examined. Sections were immersed in a drop of IKI (0.3 I to 1.5 KI to 100 water) and examined. The lesioned area was found to contain a large number of small granules, presumably starch grains, that had stained very deeply blue-black. The number of these was much greater than elsewhere in the section. A drop of 75% H_2SO_4 was then placed at the edge of the cover slip and slowly drawn under. This resulted in a deep-blue color reaction of the cellulose cell walls of all parts of the section, including the lesion, athough this was the last part to become stained even when the section was oriented so as to present the lesion first to the advancing acid. Then, within 1–3 minutes, the blue faded very rapidly from the lesion, leaving only the usual brown in this area. The loss of color was so rapid that for a time it was believed that the lesions were not producing the blue cellulose color reaction, and it was only when the lesion was kept under constant observation that the delayed staining and subsequent loss of color were seen.

Although this cellulose reaction was by no means normal, it provided further evidence that the lesion was not impermeable to microchemical reactions, and the possibilities that either a masking substance or a hydrophobic substance was present seemed unlikely.

No reaction was obtained with tests for phenolic substances. Bawden (1) states that *N. glutinosa* infected with X-virus contains "degeneration products" in the lesions, and that potato necroses contain "large amounts of breakdown products, rich in pectic substances and possibly tannins." The color of necrotic lesions would indeed suggest the presence of phenolic compounds. However, immersion of sections in 10% $FeCl_3$ up to 2 hours, did not produce the expected blue-green color reaction for such substances.

The idea that necrotic lesions contain tannins or phenolic compounds appears to be so generally accepted by workers in this field that it was felt that further attempts should be made to bring out this reaction with $FeCl_3$. A number of methods were tried:

1. In an attempt to introduce sulphonic acid groups into the phenolic compounds in the lesions, thus making them more hydrophilic and more likely to stain with $FeCl_3$, sections were immersed in fuming sulphuric acid (20% SO_3) washed in water, and placed in 10% $FeCl_3$.

2. Attempts were made to reduce any oxidized phenolic compounds present, in the hope that this would facilitate the reaction with $FeCl_3$. This was done by immersing the sections both in 2% HCl, and in 2% KOH, with zinc dust, both at room temperature and with heating. The sections were then washed and stained with $FeCl_3$.

3. Since there was a possibility that the presence of some fatty material might be interfering with the reactions, sections were dehydrated in 95% ethanol, and then immersed in chloroform for ½ hour. They were then tested for color reactions.

None of these treatments had any effect in facilitating the blue-green color reaction with 10% $FeCl_3$. Furthermore, after each treatment some of the sections were also stained with ruthenium red, without any success in staining the cell walls of the lesion cells.

Discussion.—Several interesting points are raised by the observations made here. The first is that calcium appears to be deposited in the cell walls of a narrow ring of tissue around the virus lesion, forming a firm insoluble salt with the pectic acid of the middle lamella. It is more than coincidence that this area, although it does not contain active virus, was found (9) to respire at a much greater rate than either healthy tissues, or tissues beyond the periphery of the lesion.

The fact that this band of about 50 cells around the lesions is less liable to hydrolysis by pectinase, is probably of no consequence in the natural infection. Even so, the question remains as to what mechanisms are evoked as a consequence of the virus infection that result in the deposition of calcium, and whether the resulting change in the composition of the middle lamella plays some part in localizing the virus in the lesioned area, thus preventing systemic infection.

Another point of interest is the identity of the yellow or brown materials found within the cells of the lesions. None of the methods used here gave the expected reaction of ferric chloride with phenolic compounds. It is therefore not possible to state on the basis of these data that the lesion cells contain phenolic substances or tannins.

It is difficult to reconcile the discrepancy between Bawden's statement that cells of potato necroses are "rich in pectic substances" (1) and our staining not at all with ruthenium red and only very feebly with methylene blue. It is possible that if Bawden used methylene blue for pectic substances, then the intense uptake of the dye by the cell contents in the lesions may have been interpreted as a reaction with pectic substances, since he does not mention the location of this abundance of pectic substances. It should be recalled, however, that ruthenium red, which is more specific than methylene blue for pectic materials, did not stain either the cell walls or the cell contents of the lesions.

To interpret our negative results in attempting to locate pectic substances in the lesions is difficult. If

there are no pectic substances as substrate for the pectinase, this could explain the failure of the enzyme to disintegrate the lesions, but the question remains as to how the lesions maintain their integrity (Fig. 2) if they have lost the "cement" between the cells.

The reaction of the cell walls in the lesion with iodine and sulfuric acid, in which they were the last to stain, followed by rapid loss of color, indicates that these cells are somewhat abnormal even in the cellulose composition of the wall. It is evident that the microchemistry of cells in lesions could bear further investigation.

Finally, the conversion of calcium pectate to soluble pectin in the areas surrounding the lesions several days before the onset of senility in the healthy control leaves, prompts a comparison between localized virus infections and senescence in plant tissues. Not only is such a conversion characteristic of the maturation or senescence process, but so are an increase in rate of respiration, evolution of ethylene, and changes in color (2). In virus infections also, the uninfected areas around old lesions became yellow, and the middle lamella is composed of the softer soluble pectin a considerable time before the control leaves are. The rate of respiration is greatly enhanced (8, 9), not only in the lesioned tissues but also in unlesioned cells at the periphery of the necrosis. Production of ethylene has been demonstrated (6) in a number of plants infected locally with viruses, including *N. glutinosa* infected with TMV. The similarities of the two phenomena, senescence and localized virus infections, are so striking as to warrant the suggestion that the effects of such infections can be considered as setting in motion premature senility, telescoped into an abnormally short period of several days.—Research Station, Canada Agriculture, 6660 N.W. Marine Drive, Vancouver 8, B.C.

LITERATURE CITED

1. Bawden, F. C. 1933. Infra-red photography and plant virus diseases. Nature 132:168.
2. Bonner, J. 1950. Plant biochemistry. Academic Press Inc., New York. Chapt. 9, p. 104-105.
3. Cormack, R. G. H. 1935. Investigations on the development of root hairs. New Phytologist 34:30-54.
4. Joslyn, M. A. 1950. Methods in food analysis. Academic Press Inc., New York. Chapt. 18, p. 454-460.
5. Karrer, P. 1947. Organic chemistry. Elsevier Publishing Co., New York. Chapt. 28, p. 434-435.
6. Ross, A. F., and C. E. Williamson. 1951. Physiologically active emanations from virus-infected plants. Phytopathology 41:431-438.
7. Weintraub, M. 1952. Leaf movements in Mimosa pudica L. New Phytologist 50:357-382.
8. Weintraub, M., and W. G. Kemp. 1955. The inhibiting effect of some heterocyclic and other organic compounds on tobacco mosaic virus. Can. J. Microbiol. 1: 549-559.
9. Weintraub, M., W. G. Kemp, and H. W. J. Ragetli. 1960. Studies on the metabolism of leaves with localized virus infections. I. Oxygen uptake. Can. J. Microbiol. 6: 407-415.
10. Zaitlin, M. 1959. Isolation of tobacco leaf cells capable of supporting virus multiplication. Nature 184: 1002-1003.

IMPORTANCE OF RESPONSE OF MELOIDOGYNE HAPLA TO AN AGENT FROM GERMINATING TOMATO SEEDS

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SUMMARY

Accumulation of *Meloidogyne hapla* around germinating tomato seeds is a composite effect of random nematode movement, nematode movement directed toward an agent from the germinating seed, and nematode detention at the root surface. The agent from the germinating seed increases the intensity of nematode attack, but does not govern host selection rigorously.

Plant roots usually permeate the soil rather thoroughly, and we find plant-parasitic nematodes well distributed in the soil around them. Nematode arrival at the roots by random movement seems inevitable. Kühn (8) concluded that this mechanism, alone, suffices to account for the arrival of *Heterodera rostochiensis* Wollenweber at potato roots. There is, however, evidence that a response to an agent from germinating tomato seeds plays a role in the accumulation of *Meloidogyne hapla* Chitwood around the plants (11). The experiments described here were conducted to appraise the importance of this response.

Materials and methods.—Egg masses of *M. hapla* were obtained by kneading infected tomato roots in water. They were then freed from foreign material by flotation and screening. Larvae were obtained from the egg masses placed on 4 well-separated layers of cheesecloth on a large Baermann funnel. The collected larvae were suspended in sterile distilled water and centrifuged down. Supernatant water was discarded. Washing in this manner was repeated twice. The final volume of the suspension was adjusted to a concentration of about 500 larvae per 0.01 ml, the volume used per replicate in these experiments.

Seedlings were obtained by incubating seeds at 25°C on moist filter paper—3 days for Rutgers tomato, 2 days for Kanota oats, and 1½ days for Merced rye.

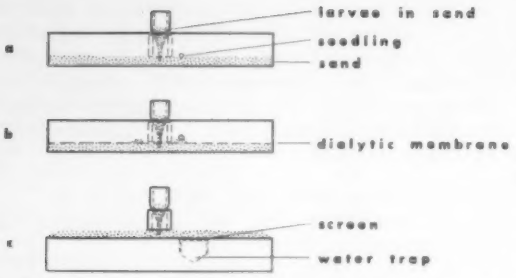

Fig. 1.—Apparatus used to study nematode accumulation. **A)** Wieser's apparatus modified for use of larvae; **B)** dialytic membrane between larvae and seedling; **C)** water trap substituted for seedling (⅔ actual size).

Nematode accumulation around seedlings was measured by Wieser's (13) method modified for use of *Meloidogyne* larvae instead of egg masses (Fig. 1-A). A dialytic membrane, through which *Meloidogyne* larvae could not pass, was interposed between larvae and seedling to measure the response to agents from the seedling (Fig. 1-B). The seedling was replaced by a boiled (to remove CO_2) distilled water trap to measure accumulation resulting solely from random movement and detention (Fig. 1-C). The trap was made by drilling a hole in a block of polymerized methyl methacrylate. A phosphor-bronze screen insert, 100 meshes per inch, prevented the moist sand from falling into the trap. Linford (10) and Wieser (13) found that *Meloidogyne* larvae accumulate around the region of root elongation. Therefore we made our trap 7 mm in diameter, about equal to the length of the region of elongation in our seedling radicles.

For each method the final assay was the same. After 24 hours at 25°C, the sand in each dish was divided into 2 halves: 1) sand from the seedling, or trap, side; and 2) sand from the opposite side. A water suspension of the sand from each half was stirred with a magnetic stirrer, the number of larvae of *M. hapla* in an aliquot was counted, and the total number was calculated.

For study of the statistical significance of differences between ratios of seedling or trap sides to the opposing sides, means and standard errors of means were determined for 28 ratios from each of the experiments (Table 2). These means necessarily differ arithmetically from ratios of means in Table 1, but relative rank is not changed.

Results and conclusions.—With the boiled distilled water trap on one side of the dish (Fig. 1-C), the number of larvae finally recovered from the trap side was about equal to the number recovered from the opposite side (Tables 1, 2).

When the tomato seedling was used (Fig. 1-A), 15 times as many larvae were recovered from the seedling side as from the opposite side (Table 2). Some of these larvae undoubtedly reach the seedling side by chance, just as they reached the trap side of the dishes involving the water well. The marked accumulation on the seedling side, however, shows that processes other than random movement must be involved.

A dialytic membrane between the tomato seedling and the larval source (Fig. 1-B) reduced accumulation on the seedling side of the dish (Table 2), but it remained statistically significant (Table 1). We conclude that this larval accumulation around the tomato seedling is, in part, a response to a dialyzable agent or agents from the tomato root, effective beyond the seedling surface. Passage through viscose-regenerated cellulose membranes with an average pore size of 24 Å indicates that the agent is not a large molecule, such as a protein or a polysaccharide. It must be a material of relatively low molecular weight. Viglierchio (12), using a different experimental procedure, showed that this agent is effective in sand or soil over a distance of at least 1 cm. The nematode responses in sand and soil were comparable, indicating that the agent was poorly adsorbed on soil colloids in the system used. Since polar substances adsorb more strongly on soil colloids than nonpolar substances, it appears that the attractive agent is relatively nonpolar, and hence poorly dissociated.

Table 2.—The ratio of larvae of *Meloidogyne hapta* on seedling, or trap, side to larvae on the opposite side of experimental dishes. Nematodes added as larvae.

Seedling or trap	Membrane	Ratio[a]	Statistical significance[b]
Rutgers tomato	Absent	15.3±2.6	X
Rutgers tomato	Present	6.3±1.0	Y
Kanota oats	Absent	4.7±0.9	Y Z
Merced rye	Absent	1.7±0.2	Y Z
Water trap	Absent	1.4±0.3	Z

[a] The mean of 28 ratios with its standard error.
[b] Duncan's Multiple Range Test (9), 1% level of significance.
Ratios followed by one or more of the same letters are not significantly different.

Table 1.—Mean numbers (with standard errors) of larvae of *Meloidogyne hapla* recovered from trap, or seedling, side and from opposite side of experimental dishes.

No. replicates	Kind of seedling or trap	Membrane	Seedling or trap side	Opposite side	LSD (1%)
28	Water trap	Absent	100±8	106±11	35
50	Rutgers tomato	Absent	235±14	38±6	39
59	Rutgers tomato	Present	136±10	35±4	29
51	Kanota oats	Absent	229±13	67±5	36
43	Merced rye	Absent	232±11	151±10	40

Bacteria were present in all these tests, with population density highest around the seedlings. There is no indication in the literature that experiments of this kind have been performed under sterile conditions. Thus far all treatments in which we have eliminated bacteria, have eliminated the response and have retarded seedling growth. In our experience, and that of Wieser (13), there is usually no nematode response when seedling growth is poor. Bacteria are known to influence nematode movement (1, 2), and they probably have a role in the responses reported here. If the attractive agent is produced primarily by bacteria accompanying the seedlings, it must be produced by a variety of bacteria. Serial dilution plates made from our experimental system showed that the principal source of bacteria was the seed. We used different kinds of seed from different sources. It is unlikely that the same bacteria would predominate in every case.

Because the dialytic membrane reduces accumulation around the seedling, we believe that accumulation is both a result of detention and a response to a dialyzable agent. Accumulation with the membrane is about half that without it, indicating that the two mechanisms contribute about equally under the conditions of these experiments. A lower rate of migration from the cone is usually obtained with the membrane, possibly because of its effect on diffusion and gas exchange.

Random movement may be involved in the response to a dialyzable agent, as in ortho-kinesis (5). Linford's (10) observation that root-knot nematodes start away from the root, then hesitate, and turn back, suggests that a reaction more efficient than ortho-kinesis is involved near the root.

Using the modified Wieser method (Fig. 1-A) we found that *M. hapla* accumulates around seedlings of Kanota oats and Merced rye (Tables 1 and 2). *M. hapla* did not invade these grain varieties in greenhouse tests in which control tomatoes became heavily infected. Concurrent experiments employing entirely different methods (12) confirm this accumulation of *M. hapla* around nonhost plants.

Viglierchio's (12) observation that tomato is attractive to *M. hapla* and *Heterodera schachtii* Schmidt, and oats are attractive to *M. hapla* and repulsive to *H. schachtii*, suggests that a chemical gradient, rather than a redox potential gradient, is operating in these systems.

Considering this evidence, we hypothesize that the root diffusates that attract or repel nematodes from a distance play only a modifying role in the host-parasite relation. They increase or decrease the intensity of nematode attack. They do not govern host selection rigorously. Nematodes are attracted or repelled by a variety of agents from a variety of sources (1, 2, 3, 6, 7, 10) not necessarily the hosts. Attraction-repulsion is only one of a series of variables (4) affecting the plant-nematode interaction.—Department of Plant Nematology, University of California, Davis.

LITERATURE CITED

1. Bergman, B. H. H., and A. J. Van Duuren. 1959. Het bietencysten aaltje en zijn bestrijding VII. Die werking van stofwisselingsproducten van sommige microorganismen op de larven van Heterodera schachtii. Mededeel. Inst. Rationeele Suikerproduct. 29:26-53.
2. Bird, A. F. 1959. The attractiveness of roots to the plant parasitic nematodes Meloidogyne javanica and Meloidogyne hapla. Nematologica 4:322-335.
3. Caveness, F. E., and J. D. Panzer. 1960. Nemic galvanotaxis. Proc. Helm. Soc. Wash. D. C. 27:73-74.
4. Chitwood, B. G. 1949. Host-parasite interrelationships of nematodes in root diseases. (Abstr.) Phytopathology 39:502.
5. Fraenkel, G. S., and D. L. Gunn. 1940. The orientation of animals. 352 p. Clarendon Press, Oxford, England.
6. Gofman-Kadoshnikov, P. B., E. W. Khoroshcho, and M. I. Smirnov. 1955. Significance of chemical factors in migration of nematodes. (In Russian.) Doklady Akad. Nauk. S. S. S. R. 103:1127-1130.
7. Klingler, J. 1959. Anziehung von Collembolen und Nematoden durch Kohlendioxyd-Quellen. Mitt. schweiz. entomol. Ges. 32:311-316.
8. Kühn, H. 1959. Zum Problem der Wirtsfindung phytopathogener Nematoden. Nematologica 4:165-171.
9. Le Clerg, E. L. 1957. Mean separation by the functional analysis of variance and multiple comparisons. U. S. Dept. Agr. ARS-20-3. May 1957. 33 p.
10. Linford, M. B. 1939. Attractiveness of roots and excised shoot tissues to certain nematodes. Proc. Helm. Soc. Wash. D. C. 6:11-18.
11. Lownsbery, B. F., and D. R. Viglierchio. 1958. Mechanism of accumulation of Meloidogyne hapla around roots of tomato seedlings. (Abstr.) Phytopathology 48:395.
12. Viglierchio, D. R. 1961. Attraction of parasitic nematodes by plant root emanations. Phytopathology 51: 136-142.
13. Wieser, W. 1955. The attractiveness of plants to larvae of root-knot nematodes. I. The effect of tomato seedlings and excised roots on Meloidogyne hapla Chitwood. Proc. Helm. Soc. Wash. D. C. 22:106-112.

EVOLUTION OF SEXUALITY AND PATHOGENICITY. II. A COMPARISON OF THE PATTERN OF SEXUALITY IN COCHLIOBOLUS VICTORIAE AND RELATED SPECIES

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SUMMARY

Studies on the segregation of ascospore isolates of *C. victoriae* indicate that compatibility is controlled by a single major gene locus. Of 537 monoascosporic isolates studied, 264 belonged to compatibility group *A* and 273 to group *a*. Monoconidial and monoascosporic isolates exhibit marked differences in degree of compatibility, as measured by the relative frequency with which isolates fail to cross with known compatible isolates.

The fact that *C. heterostrophus*, *C. carbonum*, *C. sativus*, and *C. victoriae* have similar compatibility and incompatibility mechanisms and exhibit interspecific fertility in most combinations suggests a related evolution at the species level.

In studying the evolution of sexuality and pathogenicity of related species of fungi, a thorough understanding of the kind and magnitude of genetic similarities and of genetic differences in basic mechanisms is equally important. Basic similarities in plant pathogens, not only in morphology and patterns of sexuality but also in host range, demonstrate that related species have evolved along several common lines. Species in the genus *Cochliobolus* typically demonstrate such similarities. All the species have *Helminthosporium* conidial stages and, with a few exceptions, the pathogenic species attack only gramineous hosts. Furthermore, in all the species for which the pattern of sexuality has been determined, including *Cochliobolus sativus* (Ito & Kurib.) Drechs. ex Dastur, *Cochliobolus heterostrophus* (Drechs.) Drechs., *Cochliobolus carbonum* Nelson, and *Cochliobolus nodulosus* Luttrell, compatibility or mating type is controlled by a single major gene locus (1, 2, 4, 7). Variability in degree of compatibility among isolates has been demonstrated in *C. heterostrophus* (3) and observed in the other species, indicating that additional genes influence mating capacity. It is apparent that similarities and differences in the sexual mechanisms of these related species would be reflected in the success or failure of attempted interspecific crosses. In recent studies, successful crosses have been made of *C. heterostrophus* × *C. carbonum*, *C. carbonum* × *C. sativus*, *C. heterostrophus* × *Cochliobolus miyabeanus* (Ito and Kurib.) Drechs. ex Dastur, and *C. carbonum* × *C. miyabeanus* (5). As a part of these studies, certain wild-type isolates of *Helminthosporium victoriae* Meehan & Murphy were observed to be cross-compatible with certain isolates of *C. carbonum*. When additional isolates of the 2 species were tested, some isolates of *H. victoriae* were compatible with one mating type of *C. carbonum* and others were compatible with the other. The perfect stage of *H. victoriae*, described as *Cochliobolus victoriae* Nelson (6), was then obtained by pairing these strains. Subsequent research has been concerned with determining the pattern of sexuality of the species, with particular emphasis on comparison of the compatibility mechanism of *C. victoriae* and related species. The results of those studies are reported herein.

Materials and methods.—The fungi used were 20 monoconidial isolates obtained from diseased oats from North Carolina, Mississippi, Florida, Iowa, Georgia, Texas, Louisiana, Virginia, and Wyoming.

The methods and materials used in making crosses and isolating ascospores were similar to those previously described (2). Isolates were mated by placing small pieces of mycelium on opposite sides of a section of sterile corn leaf in the center of a Petri plate containing Sach's nutrient agar. All matings were incubated continuously at 24°C.

Cultures derived from single ascospores were obtained from crosses of known compatible isolates, both conidial and ascosporic. Each monoascosporic isolate thus obtained was mated to both parental lines and to selected tester lines to determine its mating reaction. The segregation for mating reaction was determined for each cross. Matings were considered fertile if ascospores were produced.

Results and discussion.—Three hundred and sixty monoascosporic isolates obtained from 8 different conidial crosses were studied for their mating reaction. The number of ascosporic isolates obtained from any single cross ranged from 21 to 103. Of the total, 149 isolates were of one mating type, designated here as compatibility group *A*, and 162 of the other, designated as compatibility group *a*. The segregation of isolates obtained from any one specific cross never deviated appreciably from a 1:1 ratio. The compatibility type of 49 isolates remained unclassified in spite of repeated attempts to obtain fertile crosses.

The segregation of 245 monoascosporic isolates obtained from 5 different ascospore crosses totaled 115 *A* and 111 *a*. Nineteen of the isolates failed to mate with tester strains in repeated tests. The total segregation of all ascosporic isolates obtained was 264 *A* and 273 *a*. Twenty-one complete ascus progenies, including 3 eight-spored asci, were also studied for segregation of mating type. The 1:1 ratio occurred in all 3 of the eight-spored asci, whereas no more than 4 isolates of one compatibility type were obtained from asci containing fewer than 8 spores.

Concurrent studies on the mating reaction of the 20 conidial isolates indicated that additional genes influence the gene locus for compatibility. The 20 conidial isolates were mated in all possible pairs in

5 separate tests. The compatibility reactions of only 9 isolates, 2 *A* and 7 *a*, were determined from these matings. None of the 9 isolates exhibited complete intergroup fertility, in that pairings of *A* and *a* isolates frequently did not result in the production of fertile perithecia. The remaining 11 isolates failed to produce fertile perithecia in any of the conidial pairings. The 11 isolates were then paired, in 4 separate tests, with 6 *A* and 6 *a* ascospore isolates obtained from 6 crosses of compatible conidial isolates. As a result of these pairings, the compatibility of 4 additional isolates, 3 *A* and 1 *a*, was identified. In subsequent studies, the remaining 7 isolates failed to produce fertile perithecia in any pairing. The fact that these isolates are morphologically identical with the classified isolates and exhibit similar pathogenicity on susceptible varieties of oats strongly indicates that they are isolates of *H. victoriae*.

The inability of certain isolates of *C. victoriae* to mate with tester strains and the frequent occurrence of infertile matings between *A* and *a* strains indicated marked differences in degree of compatibility among isolates. These differences were more clearly demonstrated in later studies. Five *A* isolates were paired 20 times with each of 5 *a* isolates. The frequency with which isolates mated with known compatible isolates was determined. The frequency of fertile intergroup matings ranged from 48 to 77% in group *A* and from 43 to 71% in group *a*.

These results indicate that compatibility in *C. victoriae* is controlled by a single major gene locus. The marked differences in degree of compatibility within each compatibility group suggest that additional genes not only condition the amount of variability but also directly influence the major compatibility locus. The failure of certain monoconidial isolates to mate with isolates of either compatibility group demonstrates that such incompatibility or inhibitor factors can totally suppress compatibility expression. The variation among isolates, as well as the variation within isolates in repeated tests, probably results from a complex series of incompatibility factors entirely different from the "i" gene that inhibits production of perithecia in pairings of compatible strains of *C. heterostrophus*. In that species, inhibition is simply controlled and simply transmitted as a single recessive gene. The "i" gene appears quite distinct from the other factors that control variability in degree of compatibility.

In considering evolution of sexuality in related species, it is questionable whether species possessing more complex incompatibility mechanisms are more or less advanced. It is commonly believed that, in appraising the fungi as a group, the complex incompatibility systems in the higher Basidiomycetes represent the most advanced method of protection against inbreeding. If a similar analogy can be drawn at the class, family, or genus level, it might indicate that *C. carbonum* and *C. victoriae* are products of a more advanced evolution than *C. heterostrophus*. For in spite of the wide variation in degree of compatibility among strains of *C. heterostrophus*, all conidial isolates (42 *A* and 37 *a*) and all monoascosporic isolates (more than 2500) that have been studied have been easily classified into one of the two compatibility groups. Furthermore, all the 42 *A* conidial isolates have produced fertile crosses with all the 37 *a* conidial isolates on at least one occasion. Such is not the case with *C. carbonum* or *C. victoriae*.

The striking similarity in the basic pattern of sexuality, particularly in the compatibility mechanism, among *C. heterostrophus*, *C. carbonum*, *C. victoriae*, and *C. sativus* suggests that these species have certain genes in common. In the present studies on interspecific crosses in the genus *Helminthosporium*, 2 of a possible 6 crosses between these 4 species produced viable ascospores and 3 produced sterile asci. Similar compatibility mechanisms, somewhat comparable incompatibility systems, and interspecific fertility between the species suggest a related evolution. Differences in morphology and pathogenicity, somewhat different incompatibility systems, and the fact that only certain isolates of each species are cross-fertile with certain isolates of other species, usually only 1 or 2, further indicate that the 4 species ultimately evolved along different planes. It is hoped that studies with progenies obtained from these and other interspecific crosses will further clarify the similarities and differences in basic mechanisms.—North Carolina State College, Raleigh.

LITERATURE CITED

1. Luttrell, E. S. 1957. Helminthosporium nodulosum and related species. Phytopathology 47:540-548.
2. Nelson, R. R. 1957. A major gene locus for compatibility in Cochliobolus heterostrophus. Phytopathology 47:742-743.
3. Nelson, R. R. 1959. Genetics of Cochliobolus heterostrophus. I. Variability in degree of compatibility. Mycologia 51:18-23.
4. Nelson, R. R. 1960. The genetics of compatibility in Cochliobolus carbonum. Phytopathology 50:158-160.
5. Nelson, R. R. 1960. Evolution in sexuality and pathogenicity. I. Interspecific crosses in the genus Helminthosporium. Phytopathology 50:375-377.
6. Nelson, R. R. 1960. Cochliobolus victoriae, the perfect stage of Helminthosporium victoriae. Phytopathology 50:774-775.
7. Tinline, R. D., and J. G. Dickson. 1958. Cochliobolus sativus. I. Perithecial development and the inheritance of spore color and mating type. Mycologia 50:697-706.

INFLUENCE OF ENVIRONMENTAL FACTORS ON DEVELOPMENT OF INFECTION STRUCTURES OF PUCCINIA SORGHI

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SUMMARY

The influence of cellophane membranes, pH, Zn salts, light, and temperature on urediospore germination and vesicle formation *in vitro* was compared with that in the host leaf for *Puccinia sorghi*. Cellophane membranes between the urediospores and media stimulated formation of appressoria, vesicles, and hyphae from the two vesicle lobes similar to those formed in the host when subjected to the same sequence of light and temperature changes. $ZnCl_2$ and $ZnSO_4$ apparently were not essential for vesicle formation over the range of pH studied, and were toxic at concentrations, respectively, above 12 and 10 ppm. Germinating the spores on membranes 2 hours in the dark at 17°C, followed by 4 hours in 200 ft-c incandescent light at 24°C, resulted in structures similar morphologically to those formed in the host.

Introduction.—The phenomena of spore germination, penetration, and establishment of infection by cereal rust pathogens are associated with the influence of environment on the physiology directing the sequence and development of specific structures differentiated by the germ tube of the urediospore (6, 9). Production of these structures on artificial substrates by Hurd-Karrer and Rodenhiser (4) and others (1, 2, 3, 7, 8) led to an attempted evaluation of the environmental factors controlling their development *in vitro*. Influence of a contact stimulus by a membrane surface was observed in the germ-tube differentiation (1). The possible role of trace elements in the metabolism was investigated and a specific effect of zinc on the germ tubes, similar to that exerted during contact with guard cells, was concluded for some species (8). The effect of other factors, such as temperature and light, demonstrated that a single optimum temperature or light condition was not effective in efficiently completing the infection cycle. Sharp et al (7) demonstrated selective temperature requirements for spore germination (pre-appressorial development) and for the sequence of events leading to infection in the host (post-appressorial development). Complementary influence of light and temperature on the infection process was demonstrated by Emge (3). His results indicated a continuous cumulative effect of these factors, and any deviation from their optimal sequence interfered with differentiation of the germ-tube structural processes. The physiological processes conditioning the morphological sequence of development and their natural activators are relatively unexplored. The present study was designed to evaluate the effect of environments on some of these reactions in *Puccinia sorghi* Schw. Experiments with zinc chloride over a range of concentrations and at different pH levels showed no significant stimulating effect of Zn on vesicle formation in *P. sorghi*. The use of cellophane membranes in the study of the influence of temperature and light on germination and structural development of appressoria and vesicles is described and illustrated.

Materials and methods.—Cellophane membranes of medium pore size placed on media were used in all experiments. These were transferred directly to slides for examination and photographing spore structures.

Sterile 1.5% water agar containing increasing concentrations of zinc chloride from 0 to 16 ppm and adjusted to pH 5.0 to 7.0 at intervals of 0.2 was dispensed in 10-ml portions in Petri plates. The range of pH values for the substrate was based on data on urediospore germination established by LeRoux and Dickson (5). Membranes (1.5–2 cm sq) were spread evenly over the agar surface. Fresh urediospores of clonal line 3–32 of *P. sorghi*, produced under controlled greenhouse conditions on the susceptible inbred corn line P 39, were dusted over the strip onto droplets of water. Supension of small quantities of the spores in water reduced the effect of a self-inhibitor (5). The plates were incubated 2 hours in darkness at 17°C, and transferred to 24°C and incandescent light, 200 ft-c, for 4 hours, and readings then taken. Controls were parallel sets without zinc chloride and/or pH adjustment. Data on percentages of germinated urediospores and the successive structures developed were recorded from 3 replicates for each $ZnCl_2$ concentration/pH level and averaged. Water agar (1.5%) was used in Petri plates with and without membranes in the temperature and light experiments. Spore development on 3 cellophane squares or locations on the media was averaged for the mean reactions of the response to the several temperature and light sequences.

Results.—Spore germination and development of germ-tube structures were influenced by environment. Urediospores on agar and membranes, respectively, showed 90 and 95% germination and sparse vesicle formation at 17°C in total darkness. Following Emge's report (3), the optimum light intensity and differential temperatures for the development of infection structures in *P. sorghi* were studied. Urediospores on agar plates with and without cellophane strips were treated similarly. In all treatments, vesicle formation was significantly greater on cellophane than on agar.

Fig. 1–7.—Urediospore germination and vesicle formation in *Puccinia sorghi*, showing the sequence of development on cellophane membranes. Spores were incubated 2 hours in the dark at 17°C, then exposed to 200 ft-c incandescent light at 24°C. **1–2)** Germ-tube development in the dark. **3)** Primary vesicle (appressorium), formed uniformly on exposure to light. **4–5)** Secondary vesicles, differentiated at varying distances from the primary. **6–7)** Lateral lobes of secondary vesicles elongating into hyphae. ×2000.

Summary data indicated that germination and vesicle development were best from 2 hours at 17°C in total darkness followed by 4 hours under 200 ft-c incandescent light at 20 and 24°C. Prolonging either time period retarded germ-tube differentiation and resulted in tangled hyphae. Under the conditions used, temperatures above 35°C and light above 500 ft-c inhibited germ-tube differentiation.

The morphology of germ-tube infection structures was similar to that described by others (1, 3, 8). The stout germ tubes contained dense, granular, refractile protoplasm (Fig. 1). Some showed globular tips while in the dark (Fig. 2), and developed regular appressorium-like structures only after a 2–4-hour exposure to light 20–24°C. The protoplasm advanced into the primary vesicle (appressorium) and a septum was formed. A fine papilla developed from the apex of the primary vesicle that elongated into a slender tube of variable length (Fig. 3). A "secondary vesicle" developed from the apex of the tube (Fig. 4). The protoplasm advanced into these structures with increased vacuolation of the cytoplasm. The secondary vesicle soon gave rise to short, equal-sized, fine hyphae from each lobe (Fig. 5–7). Aberrations, or "atypical" structures, developed frequently, especially on the agar surface. The several structures that developed on the cellophane membrane were similar in morphology to these formed in the corn leaf.

Urediospore germination and vesicle formation on the cellophane membrane were not influenced greatly by pH and Zn salts below 10 ppm concentration of $ZnCl_2$ or $ZnSO_4$. Vesicle formation was highest (70–72%) at pH 6.4–7.0 at 4 ppm $ZnCl_2$, but the differences from the controls were not signficant. The inhibitory effect of Zn, especially on vesicle formation, appeared to increase with increase in pH, but again the interaction was not significant. Spore germination and vesicle formation dropped sharply between 12 and 16 ppm $ZnCl_2$ and 10 and 16 ppm $ZnSO_4$. At 16 ppm, germination was less than 5% with both salts.

Discussion.—The occurrence of aberrations or atypical structures is perhaps the result of imperfect differentiation conditioned by genotype or a response to unfavorable local microenvironment. Observations suggest that contact of germ tubes with a surface, sequential exposure to darkness, and low temperature followed by light and high temperature represent complementary factors responsible for successful host infection.

The influence of pH on spore germination and vesicle formation showed no significant reaction over the range studied. However, vesicle formation on the cellophane membranes without and with 4 ppm $ZnCl_2$ was highest at pH 6.4–7.0, which approximates the pH of the host cell sap. $ZnCl_2$ and $ZnSO_4$ inhibited urediospore germination and especially vesicle formation at 12 and 10 ppm, respectively.—University of Wisconsin, Madison.

LITERATURE CITED

1. Dickinson, S. 1949. Studies in the physiology of obligate parasitism. II. The behaviour of the germ tubes of certain rusts in contact with various membranes. Ann. Botany (London) 13:219-236.
2. Dickinson, S. 1949. Studies in the physiology of obligate parasitism. IV. The formation on membranes of haustoria by rust hyphae and powdery mildew germ tubes. Ann. Botany (London) 13:345-353.
3. Emge, R. G. 1958. The influence of light and temperature on the formation of infection-type structures of Puccinia graminis var. tritici on artificial substrates. Phytopathology 48:649-652.
4. Hurd-Karrer, Annie M., and H. A. Rodenhiser. 1947. Structures corresponding to appressoria and substomatal vesicles produced in nutrient solution agar by cereal rusts. Am. J. Botany 34:377-384.
5. Le Roux, P. M., and J. G. Dickson. 1957. Physiology, specialization, and genetics of Puccinia sorghi on corn and of Puccinia purpurea on sorghum. Phytopathology 47:101-107.
6. Pole Evans, I. B. 1907. The cereal rusts. I. The development of their uredomycelia. Ann. Botany (London) 21:441-446.
7. Sharp, E. L., C. G. Schmitt, J. M. Staley, and C. H. Kingsolver. 1958. Some critical factors involved in establishment of Puccinia graminis tritici. Phytopathology 48:469-474.
8. Sharp, E. L., and F. G. Smith. 1952. The influence of pH and zinc on vesicle formation in Puccinia coronata avenae Corda. Phytopathology 42:581-582.
9. Ward, H. M. 1888. Illustrations of the structure and life history of Puccinia graminis, the fungus causing the rust of wheat. Ann. Botany (London) 2:217-222.

POSSIBLE ROLE OF PECTINOLYTIC ENZYMES AND POLYSACCHARIDE IN PATHOGENESIS BY CEPHALOSPORIUM GRAMINEUM IN WHEAT

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SUMMARY

Cephalosporium gramineum produced type I polygalacturonase (PG) and pectinesterase (PE) when grown in modified Richards' solution. Small amounts of reducing sugars were detected. PG was most active and most resistant to heat at *ca.* pH 5.5. Dialysis of the fungal filtrate lowered the thermal inactivation point by *ca.* 2°C. PG production in still culture was greatest at 20°C and increased with the age of the culture. No protopectinase was detected with carrot tissue discs. The pathogen produced cellulases capable of degrading cotton, filter paper, and carboxymethylcellulose. The slow growth with these latter carbon sources suggests that cellulases are not of primary importance in the pathogenesis of the stripe fungus in wheat. *C. gramineum* produced a polysaccharide composed of glucose, mannose, and rhamnose.

Diseased tissue samples contained less moisture than those of healthy wheat at comparable stages of development, and the normal ripening of the grain was hastened. The kernels were usually smaller and with a lower starch content than kernels produced by healthy checks.

Dye uptake studies with diseased leaf cuttings revealed the inability of the striped tissue to take up the dye. The dye taken up by functional vessels could neither be carried laterally in cross veins nor diffuse into what appeared to be healthy green chlorenchyma cells on either side of the stripe; the latter phenomenon was particularly apparent during the stage of incipient striping.

Polygalacturonase, of assumed fungal origin, was demonstrated in several varieties of striped wheat but not in healthy check plants. Some correlation was observed between the susceptibility of a given variety to the stripe disease and the amount of PG detected. Both pectic and gum-like substances as well as spore masses and hyphae completely plugged some of the xylem vessels in sections of striped culms.

Heated and unheated filtrates of liquid cultures of the stripe pathogen decreased the fluid uptake of wheat plants. The decrease in fluid uptake in the excised wheat plants was correlated with the polysaccharide fraction of the fungal filtrate. It is suggested that the polysaccharide, because of its viscous nature, reduces the rate of flow of the transpiration stream until fluid movement in the vascular system is inadequate, especially in the small elements. This retarding action of the polysaccharide on fluid movement is supplemented and enhanced by the presence of fungal elements and pectin plugs in the xylem vessels. The pectin plugs are apparently derived from degradation of host tissues by fungal pectinolytic enzyme action. Vascular distress perhaps plays a primary role in the development of conditions of drought and starvation, resulting in cellular dysfunction and death of wheat plants.

Introduction.—*Cephalosporium gramineum* Nisikado & Ikata, the cause of Cephalosporium stripe of winter wheat, *Triticum aestivum* L., is a locally destructive vascular parasite of wheat in Japan (18) and the United States in Washington (2), New York (26), Montana (22), and Michigan (personal communication from R. L. Kiesling). Although the general symptom syndrome has been established (2, 18), there has been no extensive investigation of the manner in which the fungus induces the typical host responses. A toxin that inhibits seedling growth was reported (11) but was not confirmed in the United States (2). Bruehl (2) suggested that in addition to plugging of vessels with mycelium, toxic metabolites of the fungus probably play a role in pathogenesis. This conclusion was based on his observation of discolored xylem and phloem in striped wheat stems well above heavily invaded portions of infected wheat plants.

The action of pectinolytic enzymes and polysaccharides of vascular parasites of dicotyledonous plants has been extensively investigated (6, 7, 8, 9, 10, 13, 27, 28) but little such work has been done with similar diseases of monocotyledonous plants. This investigation was made to determine and characterize some of the metabolic products produced in culture by *C. gramineum* and to find relationships between these products and the disease processes in wheat plants. A preliminary report has been published (23).

Materials and methods. — *Cultural method.* — The medium employed was a modified Richards' solution (5) containing 1 g KNO_3, 2.5 g KH_2PO_4, 2.5 g $MgSO_4 \cdot 7H_20$, 0.02 g $FeCl_3$, and 5 g sucrose per liter of distilled water. The medium was adjusted to pH 5.5, dispensed in Erlenmeyer flasks in volumes not exceeding 20% of the capacity of the flasks, and autoclaved for 20 minutes at 15 psi. Inoculations were made with cultures of *C. gramineum* maintained on potato-dextrose agar. Cultures were incubated 3 weeks in the dark at 20°C. Aeration through loose cotton plugging was augmented by occasional manual shaking of the flasks. These conditions were standard except where stated otherwise. Conidia and mycelia were removed from the culture fluids by passage through filter paper and/or centrifugation.

Polygalacturonase (PG) assay.—PG was assayed by estimating the loss in viscosity of citrus pectin (Nutritional Biochemicals Corp., Cleveland, Ohio) and determining the reducing groups formed. The loss in viscosity was estimated in size-200 Fenske-Ostwald viscosimeters in a water bath at 25°C. One ml of filtrate was added at zero time to 10 ml of

buffered substrate prepared by adding 0.5*M* acetate buffer (pH 5.5) to 0.5% citrus pectin in a ratio by volume of 1 to 5. Reducing groups were determined by the Willstatter-Schudel hypoiodite method as modified by Jansen and MacDonnell (12). Galacturonic acid was determined by 1-dimensional ascending paper chromatography following the procedure described by Gothoskar et al (7).

Pectinesterase (PE) assay.—PE was determined by estimating the mg of methoxyl groups per ml of culture filtrate released in 3 hours from heated and unheated buffered enzyme-substrate solutions prepared by adding 15 ml of filtrate adjusted to pH 7.0 to a solution of 75 ml of 1.5% citrus pectin and 10 ml of 0.5*M* acetate buffer (pH 5.5) (28).

Heat of inactivation of PG.—Samples of the supernatant of standard cultures were prepared by filling *ca.* 1.5-ml-capacity vials made from 2-in. segments of ¼-in. glass tubing and sealing the ends with cork stoppers. The vials were immersed in an oil bath at a given temperature for a given period. Then the samples were removed and rapidly cooled to room temperature. After carefully removing the oil, 1.0 ml of the test material was removed with a volumetric pipette and added at zero time to 10 ml of citrus pectin buffered at pH 5.5 in accordance with the procedure followed for PG assay.

Polysaccharide isolation.—The procedure followed in isolation of polysaccharide was based on that described by Biernier (1). One liter of liquid culture grown under standard conditions was centrifuged 30 minutes at 2700 rpm to remove spores and mycelia. The supernatant was concentrated under vacuum to *ca.* 200 ml. The concentrate was treated with an equal volume of 95% ethyl alcohol, and the stringy mass of alcohol-insoluble polysaccharide was collected by twirling it around a glass stirring rod. The material was dissolved in distilled water and again recovered by treatment with alcohol. This procedure was repeated twice for partial purification of the polysaccharide. The product was given a final washing in ether and was then frozen and used as needed.

Results.—*In vitro studies.*—1) *Polygalacturonase.*—a) *PG production by isolates of C. gramineum.*—Employing the viscosimetric method of analysis of PG, filtrates of 11 isolates of the stripe fungus were assayed. The results obtained demonstrated that all of these cultures produced about equal quantities of PG under the test conditions. Chosen as the standard test organism for all studies was the Oakesdale isolate, obtained in 1956 from winter wheat near Oakesdale, Whitman County, Washington.

b) *Effect of temperature on PG production.*—Standard cultures of the Oakesdale isolate were prepared in triplicate and placed in incubators at 10°, 15°, 20°, and 25°C and occasionally shaken manually to reaerate. A set was also placed on a rotary shaker in a room maintained at 25°C. After 21 days, the filtrates were assayed viscosimetrically for PG activity. Production of PG was greatest at 20°C (Fig. 1). Since, however, PG activity at 25°C was greater under shake than still culture, it is probable that shake culture at 20°C would produce a still higher yield.

c) *Effect of substrate on PG production.*—In a comparison of sucrose and pectin as sole sources of carbon in the modified Richards' solution, pectin (0.5% solution of citrus pectin) proved relatively poor for both growth and enzyme production (PG) by the Oakesdale isolate. The breakdown and acid-reaction of pectin induced by autoclaving was circumvented by using bacteria-proof Seitz filter pads to sterilize test media. PG is concluded to be a constitutive enzyme of *C. gramineum.*

d) *Effect of pH on PG activity.*—Using aliquots of the crude filtrate of a 6-week culture of the Oakesdale isolate, PG activity was studied over a pH range of 4.2–8.0. The substrate (0.5% citrus pectin) was roughly adjusted to the given pH with HCl or NaOH and buffered with a given buffer solution in the ratio of 10 ml of pectin solution to 2 ml of buffer. Buffering in the pH range below 6.0 was obtained with 0.2*M* acetate buffer solutions, whereas 0.2*M* phosphate buffer solutions were employed between 6.0 and 8.0. The aliquot for study in the viscosimeter at 25°C was prepared by adding 1 ml of the filtrate to 10 ml of the buffered substrate at zero time. No significant variation in pH was observed during the test period. The PG activity was greatest at pH 5.4 (Table 1).

e) *Thermal inactivation of PG.*—The filtrate of a standard culture of the Oakesdale isolate was heated without modification of its pH in an oil bath for 10 minutes at a given temperature. After cooling, the filtrate was compared with an unheated control for PG activity by the viscosimetric method. The data were then plotted and the T_{50} endpoints (time required for a 50% reduction in pectin viscosity) read

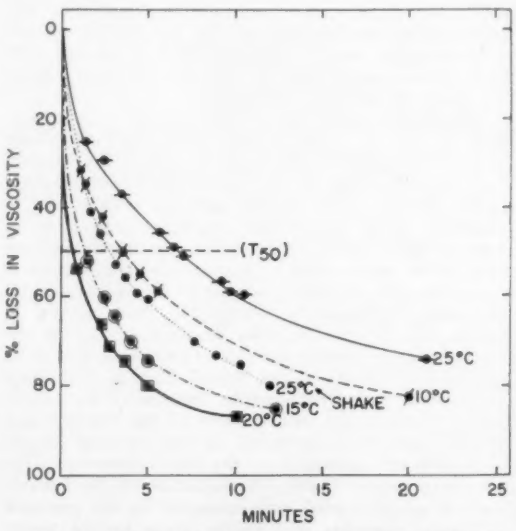

Fig. 1.—Effect of incubation temperature on polygalacturonase production by *Cephalosporium gramineum.*

directly from the graph. Thermal inactivation occured at 66–70°C in tests with different filtrates.

f) *Effect of dialysis on thermal inactivation of PG.*—Filtrate of the Oakesdale isolate grown in the standard medium for 3 weeks at 25°C was centrifuged at 20,000 rpm and the supernatant dialyzed against 4.5 L of distilled water for 48 hours at 4°C. The dialysate was discarded after 24 hours and fresh distilled water added for final dialysis. The dialyzed product was frozen in polyethylene bottles and used as needed. Both dialyzed and undialyzed supernatants were buffered to neutral pH with 0.5*M* phosphate buffer, and samples were heated at 58°, 60°, and 62°C for 10, 20, or 30 minutes. Dialysis was found to lower the temperature resistance of PG about 2°C. The medium thus affects thermal inactivation, and dialysis of filtrate is essential to obtaining reproducible data.

g) *Effect of pH on thermal inactivation of PG.*—Dialyzed supernatant of the Oakesdale isolate was buffered to final pH levels of 4.0, 4.5, 5.0, and 5.5 with 0.5*M* acetate buffers and to 6.3 and 6.8 with 0.5*M* phosphate buffers. The samples were heated 20 minutes at 60° and 62°C. All samples were then assayed for activity at the same pH and temperature, that is, pH 5.5 and 25°C. Results were then compared with the PG activity of unheated controls (25°C) that were assayed viscosimetrically at pH 5.5 shortly after buffering to the different pH levels. The data in Fig. 2 indicate that the thermal stability of PG is sensitive to pH. The pectinolytic enzyme(s) is concluded to be most stable at pH 5.5, and inactivation temperatures should be determined at this pH.

h) *Classification of PG.*—Only trace amounts of reducing sugars were detected if the incubation period of the filtrate and pectin in the hypoiodite test did not run over 4 hours. If the incubation was continued for 24 to 48 hours, however, the level of reducing sugars increased. The small increase in reducing groups during the initial stage of the degradation of pectin solutions, together with the large initial decrease in viscosity, suggests that the PG of *C. gramineum* is type I (liquefying polygalacturonases that preferentially attack pectic substances of a low degree of esterification and split the glycosidic linkages mainly at random), in accordance with the classification suggested by Deuel and Stutz (3).

2) *Pectinesterase.*—PE and PG production by the Oakesdale isolate were studied simultaneously over a three-week period of growth under standard conditions. The production of both enzymes continued to increase throughout incubation. The PE activity, expressed in terms of mg of methoxyl removed per ml of filtrate, was 2.5 at 3 weeks, whereas the PG activity, expressed in terms of the reciprocal of the time in minutes for the enzyme to reduce the viscosity of the substrate (citrus pectin) by 50%, was 0.800. Maximum production levels were not determined.

3) *Protopectinase.*—Protopectinase production was studied via the maceration test described by Echandi and Walker (4). The ability of filtrate of the standard Oakesdale culture to macerate sections of carrot root tissue was studied during 24 hours over a pH range of 3–7. None was observed. The result is supported by the observation that the growth of the Oakesdale isolate and other cultures of *C. gramineum* in liquid medium containing slices of potato resulted in abundant fungal growth without loss of integrity of the potato tissue. These results do not, however, represent conclusive proof of the inability of *C. gramineum* to produce protopectinase in wheat plants.

Table 1.—Effect of pH on polygalacturonase activity of *Cephalosporium gramineum* at 25°C.

pH	T_{20}* (minutes)	Relative activity
4.2	34.0	0.10
4.7	5.5	0.64
5.4	3.5	1.00
6.2	7.4	0.47
7.2	31.0	0.11
8.0	31.0	0.11

* T_{20} represents the time (T) required for the enzyme to reduce the viscosity of citrus pectin by 20%.

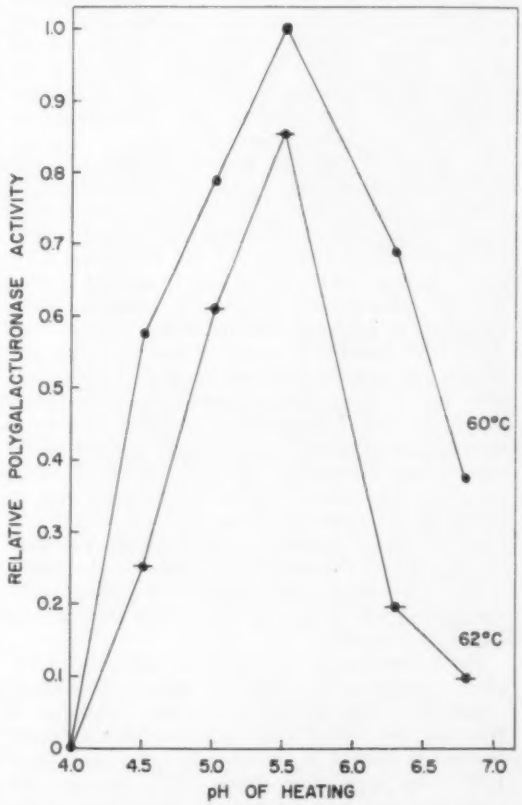

Fig. 2.—Relative polygalacturonase activity of centrifuged dialyzed culture medium of *Cephalosporium gramineum* at pH 5.5 and 25°C following incubation for 20 minutes at 60 or 62°C at different hydrogen ion concentrations.

4) *Cellulase.*—The Oakesdale isolate was grown in still culture at 20°C in the modified Richards' solution containing 0.5% concentration of either Whatman No. 1 filter paper prepared as an aqueous slurry in a Waring blender, carboxymethylcellulose (CMC 7H, supplied by Hercules Powder Co., Wilmington, Delaware), or teased fibers of absorbent cotton as sole carbon sources. The fungus grew poorly on these sources of cellulose. Cellulolytic enzyme production was assayed in a viscosimeter test similar to that used for PG assays, except that the substrate differed. One ml of filtrate from 2-month-old cultures was added at zero time to 10 ml of buffered substrate (10 ml of 0.5% CMC 7H plus 2 ml of 0.5*M* acetate buffer at pH 5.5). Cellulases were produced by the stripe pathogen on all three cellulose carbon sources. Following the classification suggested by Reese et al (21), the fungus produced C_1 cellulases when growing on cotton and filter paper and C_x cellulases when growing on CMC. In the viscosimetric assays incubated for 35 minutes, the cellulases produced on CMC 7H, filter paper, and cotton reduced the viscosity of the substrate (CMC 7H) 49.5, 73.1, and 89.0%, respectively. The results suggest that the C_1 cellulases are more efficient in the degradation of CMC 7H than the C_x cellulases. Little cellulase activity, if any, was detected in filtrate of the Oakesdale isolate grown under standard conditions with sucrose as the sole carbon source. The cellulases of *C. gramineum* are concluded to be adaptive enzymes.

5) *Polysaccharide.*—In the one instance in which yield was determined, 42 mg of the dried and partially purified polysaccharide were obtained from 1 L of liquid culture. The product was similar to the polysaccharide obtained by Biernier (1) from fungi active in the decomposition of wood and forest litter. Thus, the polysaccharide produced by the Oakesdale isolate appeared as a stringy, grayish-white, viscous mass difficult to dissolve in water. Qualitative tests for protein and carbohydrate were made both before and after hydrolysis of the polysaccharide. The sample was hydrolyzed 2 hours in 1*N* sulfuric acid in an autoclave at 15 psi. The hydrolysate was neutralized with barium carbonate to *ca.* pH 5–6. Both before and after hydrolysis, the Molisch reaction was positive, indicating the presence of carbohydrate. The Ninhydrin and Biuret reactions were negative, indicating the absence of nitrogenous constituents. A positive Fehling's test was not obtained until the polysaccharide was hydrolyzed, indicating the release of reducing sugars by hydrolysis.

The polysaccharide was analyzed chromatographically to determine the component sugars. One-dimensional descending chromatograms were developed on Whatman No. 1 filter paper with an n-butanol–acetic acid–water solvent in the ratio of 4:1:5 by volume (19). The spots were developed by spraying the chromatograms with p-anisidine reagent and heating for *ca.* 5 minutes at 100°C (17). Glucose, mannose, and rhamnose were detected by comparison with the migration and color of control sugars. According to the intensity of the spot reactions, glucose appeared to be present in the greatest concentration.

In vivo studies.—1) *Moisture content of striped wheat.—C. gramineum* has been reported (2, 18) to cause severe damage to the conductive tissue of wheat culms. Such damage could interfere with the uptake and distribution of water by wheat plants. To gain some knowledge of the water relations of striped wheat plants, determinations were made of the moisture contents of samples of Burt wheat collected in the field by severing the straw just above the roots. One hundred shoots representing the extremes of striped and healthy plants were subjectively chosen and rapidly stripped of leaf and sheath tissue. Each shoot was cut into 4 sections—the head and the upper, middle, and lower thirds of the culm. Initial weights were determined and the sections dried at 60°C to an equilibrium point represented by a final weight change of less than 1 g in 24 hours. Moisture contents were determined with samples collected from the soft-dough through the hard-dough stages.

In the soft-dough stage the moisture content was fairly uniform in all parts of the shoots of healthy plants, whereas in diseased plants, even in this stage, there was *ca.* 10% less moisture in the upper third of the culm and the head than in the lower two-thirds of the culm. In healthy plants, as the grain matured the moisture content of the head dropped rapidly; that of the culm also decreased, but to a lesser extent. The upper third of the culm contained just slightly more moisture than the head by the hard-dough stage. The loss in moisture content was faster and greater in diseased plants than in healthy plants in all parts but the lowest third of the culm.

2) *Reduced yield and protein quality.*—Previous workers have reported a reduced yield in association with the stripe disease in wheat (2, 18). The grain harvested from striped Burt, Omar, and Brevor wheats has a lower test weight and is often harder and more vitreous than grain from healthy checks. The quality of the protein in the grain of these varieties is not altered by the disease, but the starch content is reduced, resulting in a ratio of protein to carbohydrate that is higher than normal. Such characteristics are often associated with the kernels of wheat plants grown under dryland conditions.

3) *Dye uptake.*—The moisture content data suggested the presence of factors obstructing the upward movement of fluid through the culms to the heads. To detect such obstructions in the vascular pathways, dye-uptake studies were conducted with the leaves of Baart wheat plants. Leaves in various stages of stripe development were excised under water and immediately placed in test tubes containing a 1% aqueous solution of Eosin Y. Healthy leaves were treated similarly. No dye uptake was observed in striped areas of plant tissue. The width of the infected areas was greater than the chlorotic striping would indicate. The dye uptake studies indicated that malfunctioning of the vascular pathways is complete, even in the early stages of stripe formation, when

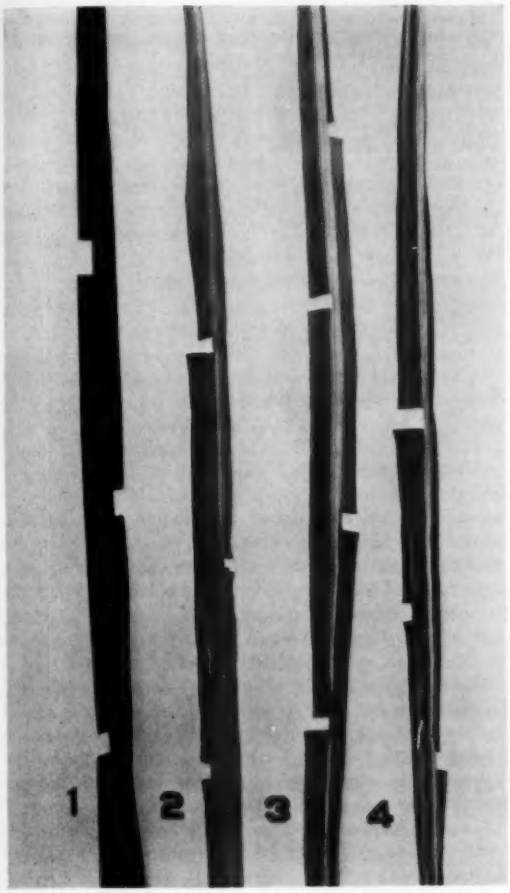

Fig. 3.—Effect of striping on the lateral passage of dye (0.5% acid fuchsin) through the vascular system of excised wheat leaves. **1)** Healthy green leaf with segments excised to force lateral passage of dye illustrates unimpeded dye movement. **2)** Moderate degree of striping allowing some diffusion of dye, especially in the upper areas of the stripe. **3, 4)** Severely striped leaves in which the major stripes effectively prevented the lateral passage of dye.

chlorosis is discontinuous, with a mosaic-like pattern, and the majority of the cells are still green. Unaffected areas of the striped leaf and healthy leaves took up the dye rapidly. The cells of the healthy tissue were deeply stained.

These experiments also indicated that the striped areas blocked the lateral movements of dye. To check the impermeable nature of the striped zone, striped leaf samples were excised under water and segments of the leaf were removed at various levels to block the upward flow artificially and to force a lateral movement of dye. The results (Fig. 3) showed that the lateral movement of dye proceeded readily in healthy leaf samples but was prevented in striped leaves. Such evidence indicates that fungal action results in a plugging of the main vascular channels and the lateral veins as well.

4) *Polygalacturonase production.*—A determination of the pectinolytic enzymes of *C. gramineum* in striped wheat was considered essential to demonstrate their possible relationships to the disease process. Accordingly, a comparison was made of the PG produced *in vivo* in 6 varieties of winter wheat—Columbia, Brevor, Elgin, Itana, Omar, and Burt. Healthy and heavily striped specimens of each wheat variety were collected in the field by cutting just above the roots. The samples were immediately brought to the laboratory, and the leaves, sheaths, and heads were removed and discarded. The culms were cut into short sections, and 26 g of each sample was ground in a small kitchen-type grinder. The grindings and expressed juices were caught on a sheet of waxed paper. The plant juices were then collected by squeezing the grindings through cheesecloth. The tissues were moistened with distilled water during this process, and squeezing and extracting were continued until 8 ml of plant juice was obtained from each sample. One ml of a 10% solution of a chelating agent (Versene Powder supplied by the Dow Chemical Company, Midland, Michigan), adjusted to pH 5.9 with HCl, was added to the crude extract to bind such ions as calcium, which were thought to be responsible for the rapid gelation of the buffered pectin substrate observed in preliminary viscosimetric studies. Particulate matter was removed by centrifuging 15 minutes at 2000 rpm. The clear supernatant was decanted and employed in a viscosimetric analysis of PG as described for fungal filtrate. This procedure was employed for the PG assay of sections of healthy

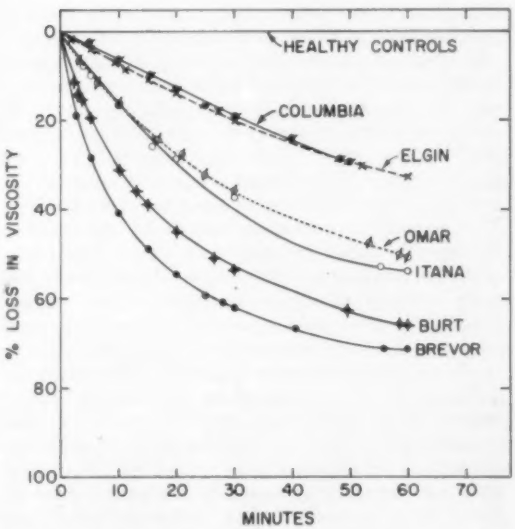

Fig. 4.—Results of polygalacturonase assays of the culm tissues of healthy and striped wheat varieties during the soft-dough stage.

and diseased wheats of all six varieties, comparing them in the milk and soft-dough stages of development.

No PG production was detected in juices of the healthy check plants used as controls. In contrast, the juices of all the varieties infected with the stripe fungus contained PG. The PG was postulated to be of fungal origin. The PG concentration increased with the maturation of each variety, being especially marked in Brevor. Some correlation was found between the PG concentration of the juices of a given variety and its relative susceptibility to the stripe disease (Fig. 4). Bruehl (unpublished data), on the basis of yield reductions, classified Burt and Brevor as highly susceptible, and Elgin, Omar, and Columbia as less susceptible.

5) *Vascular plugging.*—If the production of PG in infected wheat plants is a significant part of disease development, pectin plugs might be formed within the vascular system. With this possibility in mind, free-hand cross sections were made of infected culms. The sections after staining for 60 minutes in 1:5000 ruthenium red solution showed partial and complete pectin plugs within some of the xylem vessels. Thus, additional proof of the significant role played by PG was obtained. On the other hand, some of the vascular pathways were partially filled with mycelium, while others were plugged with a yellow to dark amorphous material, which did not take up the ruthenium red dye. At least three mechanisms of vascular plugging are involved in disease development.

6) *Fluid-uptake inhibition.*—The relative contribution of pectinolytic enzymes and polysaccharide in the stripe disease of wheat was as yet unclear. An experiment was therefore performed to detect their relative influences on the uptake of fluid by excised wheat plants. Standard liquid culture filtrates of the Oakesdale isolate were divided into alcohol-soluble and insoluble fractions. Polysaccharide (Fraction I) was obtained as previously described. The cloudy alcoholic solution from which Fraction I was removed was centrifuged, and the centrifugate, after decantation of the supernatant, was removed with a small volume of distilled water and stored in the frozen state. The alcohol-insolubles removed by centrifugation are herein referred to as Fraction II. The alcoholic supernatant containing the alcohol-solubles was taken to dryness *in vacuo*. The rather sticky substance was redissolved in distilled water and again brought to dryness to remove the alcohol completely. The residue was taken up in a small volume of distilled water and frozen for future use. The alcohol-solubles are herein referred to as Fraction III.

The day before a test in wheat, the fractions and the unfractionated supernatant were allowed to thaw and a given aliquot of each fraction was removed and diluted with distilled water to reestablish the approximate concentration of materials existing prior to the fractionation procedure, that is, to the same concentrations present in the original culture filtrate. The unfractionated supernatant was used undiluted. Each sample was adjusted to pH 5.5, divided into equal volumes, and placed in Erlenmeyer flasks with aluminum foil caps. One set was heated in an autoclave for 15 minutes at 15 psi. Each sample was then assayed for pectinolytic enzyme content by means of the standard viscosimetric procedure previously described. The samples were stored temporarily at 5°C.

Table 2.—Effect of fractions of the supernatant fluid of a *Cephalosporium gramineum* culture on the fluid uptake of excised wheat plants in 24 hours.

Fluid adjusted to pH 5.5	Usual range[a]
Distilled water	100
Modified Richards' solution	77–87
Pectinol 100D	
Heated	34–37
Not heated	78–93
Unfractionated supernatant	
Heated	35–45
Not heated	44–50
Fraction I (alcohol-insoluble)	
Heated	30–47
Not heated	44
Fraction II (alcohol-insoluble)	
Heated	66–81
Not heated	64–85
Fraction III (alcohol-soluble)	
Heated	74–131
Not heated	62–88

[a] Based on 4 determinations.

Wheat shoots of the variety Baart were excised at the base and quickly placed in tap water. Plants in the boot stage of development were chosen. The wheat cuttings were trimmed under water and immediately placed in test tubes (one cutting per tube) containing the various heated and unheated test solutions and the appropriate controls. The tests with the wheat cuttings were allowed to proceed under greenhouse conditions with supplemental fluorescent lighting at a temperature of *ca.* 75°F. At 24 hours the fluid uptake of each wheat cutting was measured. So differences in uptake from variations in sizes of cuttings could be equated rapidly and conveniently, the tissue volume of each cutting was determined by displacement in water containing a small amount of a wetting agent. Fluid uptake was then determined in terms of ml of test fluid per cc of tissue volume. Using water as 100, a relative scale of fluid uptake was calculated for several repeated tests.

The unheated Pectinol 100D and the unheated fungal supernatant were the only solutions containing active pectinolytic enzymes. Measured viscosimetrically, the supernatant contained 3 times as much enzyme as the Pectinol 100D solution.

The data in Table 2 indicate that the fraction containing polysaccharide, Fraction I, reduced the fluid uptake of the wheat cuttings about to the same degree as the unfractionated supernatant. In contrast, Fractions II and III allowed a fluid uptake extending into and beyond the levels observed with modified Richards' solution and distilled-water controls. The fact that Pectinol 100D interfered with uptake only

when heated, indicates that pectinolytic enzyme activity does not exert any measurable effect under the conditions of this experiment. The decrease in fluid uptake when Pectinol 100D and the unfractionated supernatant are heated cannot be explained. The decrease may be due to the clogging of the vascular elements by heat-coagulated enzymes. Since the fractionation processes destroyed pectinolytic enzyme as measured viscosimetrically, the unheated unfractionated supernatant served as a check on the combined action of polysaccharide and pectinolytic enzyme. Under the conditions used, no combined effects could be detected. Therefore, assaying the importance of pectinolytic enzymes in the disease process by the fluid uptake method would not seem possible with the chemical procedures of this study. Nevertheless, the data obtained with Fractions II and III suggest that the polysaccharide of Fraction I causes the main interference to fluid uptake.

During these studies, the freezing-point depressions of the fluids were determined and the potential osmotic pressures calculated (14). The modified Richards' solution and unfractionated supernatant had high osmotic concentrations, whereas all other fluids had little osmotic concentration, if any. Thus, no correlation could be found between the osmotic pressure of a given fluid and its uptake by a wheat cutting. Although high osmotic concentration alone can reduce fluid uptake by exosmosis, the results with the polysaccharide fraction (very low osmotic concentration) cannot be explained on this basis.

Discussion.—Although the direct mechanisms active in pathogenesis were the immediate objectives of this study, some of the findings apply to other facets of the disease problem as well. Considered below are factors relating to survival in host debris, penetration of the unwounded host, adaptation to life within the xylem vessels, and the possible harmful role of some of the fungal metabolites in the wheat plant.

Most species of the form genus *Cephalosporium* occurring in association with plants or plant parts are members of the soil microflora or are facultative parasites. *C. gramineum* is known to have some ability to survive in wheat refuse or in the soil. It persists in quantity from one wheat crop to the next in the absence of a host for at least a year or two in Washington. Wheat–fallow–wheat is a common cultural sequence, and heavy stripe can occur in such a system even when 13–15 months intervene between the crops. The pathogen persists at least 2–3 years in dried infested host materials stored in the laboratory. These observations establish a limited period of survival with no living host.

Its ability to grow on media containing no organic nitrogen and its use of a wide variety of carbohydrates (23) would indicate that straw, even though weathered, serves adequately as food. This is indicated by the fact that infested straw is a source of inoculum in the field (2, 18). Many observations of this laboratory suggest that, though a slow grower and probably a poor competitor in colonizing refuse, the fungus possesses antibiotic materials that surely aid in maintaining its possession of infested refuse. Perhaps the excretion of polysaccharide into the aqueous materials surrounding the spores in the spore heads could contribute to the survival of conidia. Bacterial longevity is often increased by slimes of a polysaccharide nature (13).

C. gramineum seldom penetrated to the vascular elements and rarely established a systemic infection in greenhouse trials in which the roots were not wounded (Bruehl, unpublished). The low level of cellulolytic enzyme production, the failure to produce protopectinase in culture, the paucity of lysigenous cavities in diseased wheat stems, and the lack of highly developed appressoria of the *Collectotrichum-Gloeosporium* type all indicate a limited ability to penetrate the underground tissues to the depth of the vascular elements. In Washington the disease is most prevalent on winter cereals, usually on soils of high winter moisture content, suggesting that frost-heaving of the soil or some other physical injury aids penetration in nature. Once within the metaxylem vessels of the host, invasion proceeds rapidly, presumably the result of conidial transport in the xylem stream and multiple local coalescing infections within the vessels (2, 18).

The simple nutritional requirements of *C. gramineum* adapt it to the xylem fluids of wheat. The fungus tolerates a wide pH range (18), so that slightly acidic xylem contents (16) should not hinder its activities. The pectinolytic enzymes (PG) of this fungus possess their greatest activity and stability at pH 5.5. The fungal polysaccharide is likewise stable at this pH. Though not considered anaerobic (18), *C. gramineum* tolerates low oxygen tensions. Oxygen is not considered to be a limiting factor to the growth of the pathogen in the wheat plant since the oxygen tension in wheat would be expected to be at least as high as that obtained in still culture, if not higher. The pathogen's adaptation to mineral salt nitrogen, use of many carbohydrates, growth in liquid culture, and the small size of its spores all contribute to its life within xylem vessels.

The lack of living protoplasm in mature xylem vessels would seem to provide this fungus minimum contact with the vital processes of the host while it is in the parasitic state. For this reason the authors feel justified in rather freely extending *in vitro* findings to *in vivo* interpretation. PG was produced abundantly *in vitro* and *in vivo*. No attempt was made to detect other metabolites *in vivo*. Nevertheless, this reasoning—that xylem parasites are essentially growing in an "*in vitro*" medium, little affected by vital host activities—should not be carried too far. If this reasoning is sound, parasites such as this should thrive within a wide range of hosts, since the xylem contents of almost any plant must contain a certain minimum of nutrients.

The lack of vertical conduction of dye in the striped tissue of wheat leaves is evidence of impairment of the normal functioning of vascular tissue.

The inability of dye to pass laterally through the striped tissue demonstrates not only the blockage of lateral venations but also the impervious nature of the associated chlorenchyma, epidermis, and parenchyma cells. The evidence that the affected area is wider than the chlorotic striping suggests that the malfunctioning of the vascular system and the alteration in the normal permeability of the cell walls and/or membranes of the associated cells precedes chlorosis. It is postulated that the main and tributary lateral vascular channels become gradually affected, thereby reducing the water and nutrient intake into affected tissues until a point of incipient chlorosis is reached. Probably not all the lateral veins are involved at the same time. Therefore chlorotic spotting of the mosaic type as described by Nisikado et al (18) and Bruehl (2) could occur up and down the chlorenchyma adjacent to infected major bundles.

Fungal polysaccharide and pectinolytic enzymes were the only harmful metabolites discovered in this study. No substances highly toxic to wheat were found; neither were auxins (by the pea test) nor ethylene (by the tomato epinasty test). The cause(s) of the phloem necrosis and the browning of the veins was not found. Although some suggestions have been made concerning the development of the chlorotic striping, it is clear that many facets of the stripe disease remain to be explained.

Polysaccharides (or gum-like substances) and pectinolytic enzymes have been suggested to act either directly or indirectly to plug the vascular elements of dicotyledonous plants, thereby inducing symptoms of wilt (7, 9, 10, 13, 15, 20, 24, 25, 27). The finding of pectin plugs in the xylem vessels of severely striped culm tissue together with the demonstration of PG in six varieties of winter wheat and the partial correlation between the amount of PG found and the relative degree of susceptibility of the given wheat varieties supports the theory that the pectinolytic enzymes act in wheat in a manner similar to that claimed in tomato plants infected with *Fusarium oxysporum* f. *lycopersici* Snyd. & Hans. (27). On the other hand, wheat plants are susceptible to attack during the later stages of maturation, when tissues are harder and secondary cell walls containing relatively little pectin are well-developed, whereas the opposite condition exists in young tomato plants susceptible to the Fusarium wilt disease (7). In line with this reasoning, it is postulated that during the early stages of the development of wheat seedlings an inhibitor is present that retards the growth of the stripe pathogen.

The finding that the polysaccharide fraction produced in cultures of *C. gramineum* is the only fraction that will reduce fluid uptake in wheat cuttings, whether heated or not heated, together with the observation of gum-like masses in the xylem vessels of diseased culm tissues, indicates the possible importance of polysaccharides in pathogenesis. Although the gum-like substances have not been analyzed microchemically, *C. gramineum* was found to produce a polysaccharide in culture under nutritional and environmental conditions that suggest that the compound could be produced *in vivo*. The viscous nature of the polysaccharide suggests an ability to increase the normal viscosity of the transpiration stream and eventually to plug the channels to such an extent that the water and nutrient supply is reduced to a critical level, with drought and starvation of the wheat plant resulting. A similar theory was advanced by Husain and Kelman (9) to explain the wilting of tobacco plants infected with the Granville wilt pathogen, *Pseudomonas solanacearum* E. F. Sm. The conidial and hyphal masses, together with the pectin plugs in the vessels, are regarded as contributing to the primary action of the polysaccharide.—Departments of Plant Pathology and Agricultural Chemistry, Washington State University, Pullman.

LITERATURE CITED

1. Biernier, B. 1958. The production of polysaccharides by fungi active in the decomposition of wood and forest litter. Canad. J. Microbiol. 4:195-204.
2. Bruehl, G. W. 1957. Cephalosporium stripe disease of wheat. Phytopathology 47:641-649.
3. Deuel, H., and E. Stutz. 1958. Pectic substances and pectic enzymes. Advances in Enzymol. 20:341-382.
4. Echandi, E., and J. C. Walker. 1957. Pectolytic enzymes produced by Sclerotinia sclerotiorum. Phytopathology 47:549-552.
5. Fergus, C. L., and D. C. Wharton. 1957. Production of pectinase and growth-promoting substance by Ceratocystis fagacearum. Phytopathology 47:635-636.
6. Gothoskar, S. S., R. P. Scheffer, J. C. Walker, and M. A. Stahmann. 1953. The role of pectic enzymes in Fusarium wilt of tomato. Phytopathology 43: 535-536.
7. Gothoskar, S. S., R. P. Scheffer, J. C. Walker, and M. A. Stahmann. 1955. The role of enzymes in the development of Fusarium wilt of tomato. Phytopathology 45:381-387.
8. Green, R. J., Jr. 1954. A preliminary investigation of toxins produced in vitro by Verticillium albo-atrum. Phytopathology 44:433-437.
9. Husain, A., and A. Kelman. 1958. Relation of slime production to mechanism of wilting and pathogenicity of Pseudomonas solanacearum. Phytopathology 48:155-165.
10. Husain, A., and A. Kelman. 1958. The role of pectic and cellulolytic enzymes in pathogenesis by Pseudomonas solanacearum. Phytopathology 48:377-386.
11. Ikata, S., and I. Kawai. 1937. Studies on the stripe disease of wheat (translated title). Bull. Agr. Expt. Sta. Okayama, Japan. Extra No., 111 p. (Abstr. in Rev. Appl. Mycol. 17:593. 1938.)
12. Jansen, E. F., and L. R. MacDonnell. 1945. Influence of methoxyl content of pectic substances on the action of polygalacturonase. Arch. Biochem. 8:97-112.
13. Leach, J. G., V. G. Lilly, H. A. Wilson, and M. R. Purvis, Jr. 1957. Bacterial polysaccharides: The nature and function of the exudate produced by Xanthomonas phaseoli. Phytopathology 47:113-120.
14. Loomis, W. E., and C. A. Shull. 1937. Methods in plant physiology. 1st ed., McGraw-Hill Co., Inc., New York. 472 p. (see p. 76-77, 334-337).
15. Ludwig, R. A. 1952. Studies on the physiology of hadromycotic wilting in the tomato plant. Macdonald Coll. Tech. Bull. 20.

16. Martin, S. H., M. W. Rea, and J. Small. 1926. The reaction of plant tissues. Brit. Assoc. Advance Sci. Rept. 94:410.

17. Mukherjee, S., and H. C. Srivastava. 1952. Improved spray reagent for the detection of sugars. Nature 169:330.

18. Nisikado, Y., H. Matsumoto, and K. Yamauti. 1934. Studies on a new Cephalosporium, which causes the stripe disease of wheat. Ber. Ohara Inst. landwirtsch. Forsch., Kurashiki, Japan 6:275-306.

19. Partridge, S. M. 1948. Filter paper chromatography of sugars. I. General description and application of qualitative analysis in apple juice, egg albumin, and foetal blood of sheep. Biochem. J. 42:238-250.

20. Pierson, C. F., S. S. Gothoskar, J. C. Walker, and M. A. Stahmann. 1955. Histological studies on the role of pectic enzymes in the development of Fusarium wilt symptoms in tomato. Phytopathology 45:524-527.

21. Reese, E. T., R. G. H. Siu, and H. S. Levinson. 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. 59:485-497.

22. Sharp, E. L. 1959. Two previously unreported fungi on cereals in Montana. Plant Disease Reptr. 43: 12-13.

23. Spalding, D. H., G. W. Bruehl, and R. J. Foster. 1959. Substrate utilization and pectolytic and cellulolytic and polysaccharide production by Cephalosporium gramineum. (Abstr.) Phytopathology 49: 551.

24. Struckmeyer, B. Esther, C. H. Beckman, J. E. Kuntz, and A. J. Riker. 1954. Plugging of vessels by tyloses and gums in wilting oaks. Phytopathology 44:148-153.

25. Thomas, C. A. 1949. A wilt-inducing polysaccharide from Fusarium solani f. eumartii. Phytopathology 39:572-579.

26. Tyler, L. J., and L. E. Dickens. 1957. Cephalosporium leaf stripe disease of winter wheat. Plant Disease Reptr. 41:384.

27. Waggoner, P. E., and A. E. Dimond. 1955. Production and role of extracellular pectic enzymes of Fusarium oxysporium f. lycopersici. Phytopathology 45:79-87.

28. Winstead, N. N., and J. C. Walker. 1954. Production of vascular browning by metabolites from several pathogens. Phytopathology 44:153-158.

THE NUTRITION OF HELMINTHOSPORIUM CARBONUM RACE 1 IN RELATION TO PARASITISM OF CORN

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SUMMARY

The nutrition of *Helminthosporium carbonum* was investigated by varying the nutrients in the basal medium, by assaying plant sap and extracts of resistant and susceptible hosts for possible inhibitory substances, and by analyzing the resistant and susceptible hosts for carbohydrate content and free amino acids. *H. carbonum* grew well on a variety of carbon and nitrogen sources and in the absence of added growth substances. Dry weight of mycelium indicated that growth was related to the amount of carbohydrate and nitrogen available in the medium and to nitrogen source and pH of the medium. The organic source of reduced nitrogen affected quantity of growth and pH of the culture filtrate; both reduced and oxidized forms of nitrogen were used by the fungus. *H. carbonum* was used to assay the expressed sap of host plants for a possible chemical substance causing resistance, but the assays were complicated by different levels of carbon and nitrogen sources and kind of nitrogen source. Any growth-inhibiting or promoting substance might have been masked by variations in these factors. Resistance could not be correlated with levels of sugars or with free amino acids in healthy plants. Growth of the pathogen *in vitro* was inhibited by alcoholic extracts of diseased susceptible plants, but not by extracts of diseased resistant plants or by extracts of uninfected resistant and susceptible plants.

Helminthosporium leaf spot of corn is not of serious economic importance since most lines of corn and their hybrids resist invasion by *Helminthosporium carbonum* Ullstrup race 1, the causal organism. Only certain inbred lines of corn such as K44 are susceptible, and Ullstrup (16) has shown that the susceptibility of this line is inherited as a monogenic recessive factor; line K41 is resistant. Since a single gene pair controls resistance and susceptibility, a study of the physiology of resistance of this material may contribute to understanding of the nature of disease resistance. Little is known about the physiology of the fungus that causes the disease or about interaction between the fungus and the host; therefore, the nutritional requirements and growth of the fungus were investigated in relation to its parasitism of corn.

Materials and methods.—The fungus was isolated from infected corn grains and carried in stock cultures on potato-dextrose agar (PDA). Storage under oil kept such cultures viable as long as 3 years without loss of pathogenicity. Other workers (3) have kept cultures of *Helminthosporium* spp. alive with this

Table 1.—Composition of the basal medium.[a]

Element	Amount (g/L)	Source	Amount (g/L)
C	4.0	D-glucose	10
N	0.427	L-asparagine	2
Mg	0.049	$MgSO_4 \cdot 7H_2O$	0.5
S	0.065		
K	0.287	KH_2PO_4	1.0
P	0.228		

[a] Two ml of stock solution containing the following ions as the sulfate salt were added to each liter of medium: Fe^{+++} 0.2 mg/L; Zn^{++} 0.2 mg/L; Mn^{++} 0.1 mg/L.

method for over 7 years. In general, cultural methods outlined by Lilly and Barnett (9, 10) were used as far as was practical. A liquid culture technique using 125-ml Erlenmeyer flasks was employed for most of the *in vitro* nutritional studies. Each flask contained 20 ml of a glucose–asparagine–inorganic salts medium (Table 1) with the acidity usually adjusted to pH 6 and without added vitamins unless otherwise indicated. The inoculated flasks were placed in an incubator at 29°C for varying periods, depending on the nature of the experiment. Growth of the fungus was measured by filtering the mycelium on previously dried and weighed filter papers. These were then dried 48 hours at 90°C and reweighed, and the difference in weight was taken as the weight of the mycelium.

Seed of corn inbred lines was obtained from the Agricultural Alumni Seed Improvement Association, Lafayette, Indiana. Seedling plants 3–4 weeks old grown in soil on a greenhouse bench were harvested for expression of juice and for amino acid determinations. Amino acid determinations made use of the plant extraction procedure of Awapara (1) and the qualitative paper chromatographic procedure of Levy and Chung (8). Not many amino acids were detected by this method. Further concentration of the extract led to "overloading" of the filter paper with some of the amino acids to the extent that those present in smaller amounts were not identifiable. Quantitative analyses for sugars were made colorimetrically according to Nelson's adaptation of Somogyi's method (12). The leaves and stems of seedling corn plants were extracted for 24 hr in Soxhlet extractors using 80% ethanol as the extractant. The ethanolic extracts were evaporated to dryness and taken up in distilled water. Equal volumes of 5% $ZnSO_4 \cdot 7H_2O$ and 0.3N $Ba(OH)_2$ were added to the diluted extract. This mixture was filtered and the sugar determinations made on the resulting deproteinized and decolorized filtrate.

To obtain material for the extracts of diseased and healthy resistant and susceptible hosts, plants of K44 and K41 three weeks old were inoculated by atomizing onto the leaves a homogenized agar culture of *H. carbonum*. Several plates of the organism growing on PDA for five days were placed in a Waring blender with 200 ml of distilled water and homogenized for 2 minutes. This mixture was then sprayed on the plants completely wetting all foliage. The plants were then placed in a fog room in the greenhouse for 36 hours. Seven days after inoculation the plants were harvested. The infected areas of the leaves were cut out and about 40 g of material for each treatment was extracted in ethanol and assayed as outlined by Kuć et al (7). An agar medium was made using these extracts and after spot inoculating with *H. carbonum* the diameter of the colony was measured each day for five days.

Results.—*Preliminary experiments.*—In preliminary experiments a food press was used to express juice from the ground up aerial portion of 3–4-week-old corn plants grown in the greenhouse. The expressed juice was either solidified with agar or used directly in a liquid medium (13). Sterilization was effected by autoclaving or filtering (14). Growth of the fungus was not consistently inhibited, as expected, by the expressed juice of resistant corn (Table 2). The variability led to an investigation of factors that might be responsible. Diluting the expressed plant sap with the basal medium gave no differences in yield of mycelium, with either the sap of resistant or susceptible plants, although yields were higher than when the basal medium was used alone. Yeast extract incorporated into the medium at 0.2%, 1.0%, and 2.0% yielded more mycelium than did the basal medium, but still not as much as corn sap. Increasing the glucose concentration from 1% to 2% increased mycelium weight by 70% over that produced on the basal medium and 2% glucose plus 2% yeast extract increased the yields by 125% at the end of 8 days growth.

Studies with vitamins.—Since yeast extract and corn sap stimulated growth and both should contain vitamins not present in the basal medium, experiments were carried out with 4 vitamins—thiamin, biotin, inositol, and pyridoxine—for which fungi are most commonly deficient, added to the medium (5, 9). Vitamin-free casein hydrolysate was substituted for asparagine as a nitrogen source in the basal medium, and the medium was heated with activated charcoal to remove any vitamins present in the chemicals before the vitamins and minor elements were added. The

Table 2.—PH of culture filtrate at time of harvest and dry weight of mycelium of *Helminthosporium carbonum* race 1 produced on undiluted expressed corn sap from greenhouse-grown plants.[a]

Age of culture	K44 Seitz filtered		K44 autoclaved		K41 Seitz filtered		K41 autoclaved	
	mg	pH	mg	pH	mg	pH	mg	pH
3 days	126.4	5.6	126.5	7.0	140.5	7.3	64.7	7.6
6 days	179.5	8.9	147.5	8.9	159.2	8.8	159.6	8.9
10 days	133.1	9.2	109.0	9.2	101.0	9.1	114.1	9.1

[a] Data are averages for 3 flasks for each treatment and interval. Initial pH of K44 sap was 5.1, and of K41 sap was 5.4.

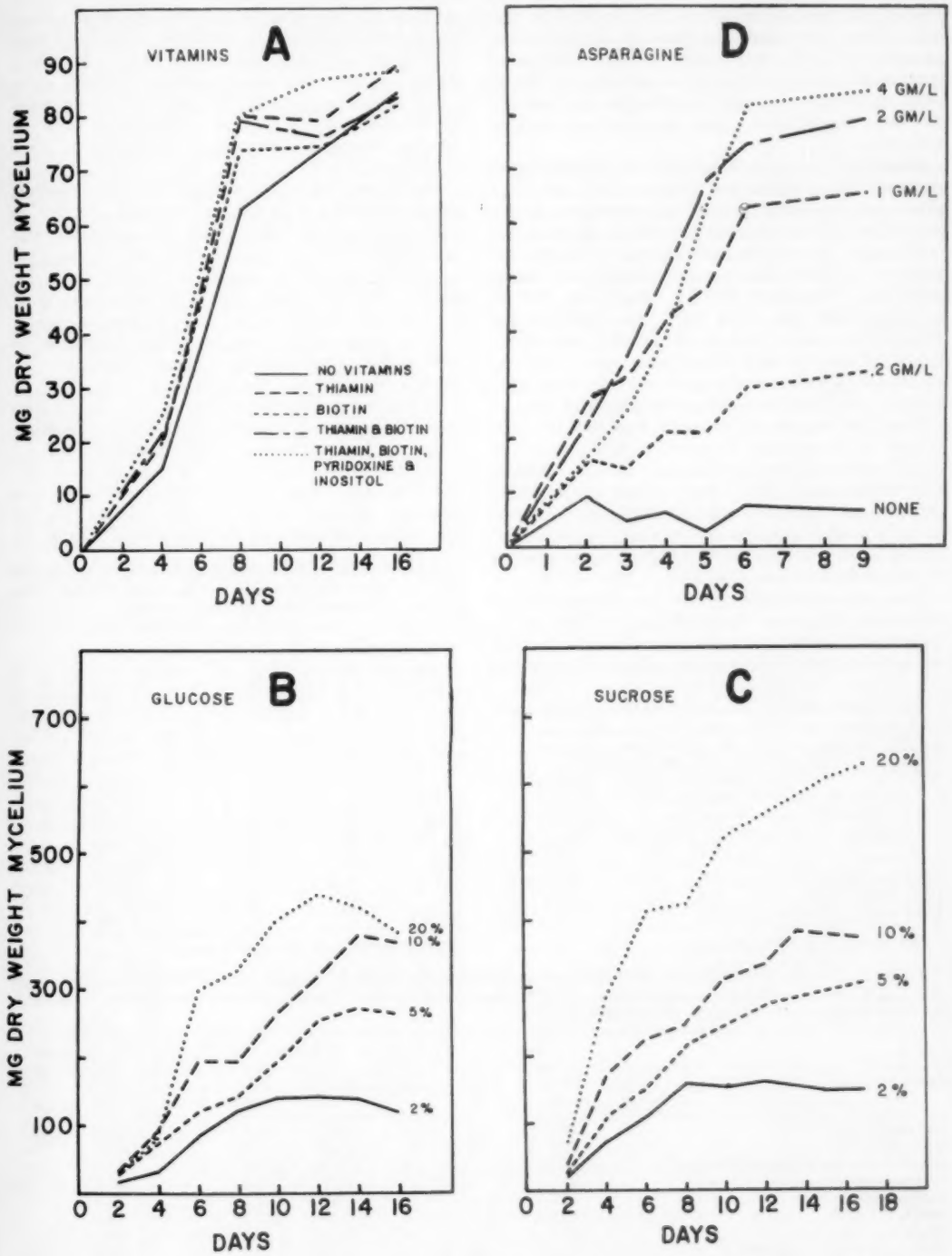

Fig. 1.—Growth of *Helminthosporium carbonum* on liquid media: **A)** on casein hydrolysate-glucose medium, with and without vitamins, as indicated; **B)** on various concentrations of glucose; **C)** on various concentrations of sucrose; **D)** on the basal medium with various concentrations of asparagine.

results in a typical experiment are presented in Fig. 1-A. There was some increase in growth when thiamin and biotin were added and the addition of inositol and pyridoxine caused a further small increment of growth. In other experiments not reported this latter small increase was found to be caused by pyridoxine.

Studies with sugars.—When the fungus was cultured on media into which was incorporated each of 6 sugars in equimolar carbon concentrations at the concentration used in the basal medium, all six sugars —galactose, glucose, sucrose, maltose, fructose, and mannose—yielded about equal dry weights of fungus mycelium. The respective dry weights on the 8th day were 82.0, 80.3, 78.3, 77.4, 75.1, and 72.4 mg. These values were averages of mycelial dry weight from 5 flasks for each sugar and were similar to results of one other such experiment. In addition to varying the kind of sugar, the concentrations of glucose and sucrose were varied from 1% to 20%. Growth of *H. carbonum* increased as the concentration of the carbon source was increased up to the highest concentration used (Fig. 1-B,C). Yield was higher on sucrose than glucose, probably because the sugars were not added in equimolar concentrations with respect to carbon. Results of only one of two identical experiments are presented.

Since the concentration of the carbon source caused such great differences in growth rate of the fungus, quantitative analyses of sugars were made from 80% ethanolic extracts of field-grown and greenhouse-grown plants of the two inbred lines. The greenhouse-grown plants were 3–4-week-old seedlings which had been growing in soil on a greenhouse bench. The field-grown corn grew for several weeks in field soil which was irrigated as needed. All plants were harvested about noon. Only in extracts of field-grown corn was there a consistent difference between the two lines (Table 3).

Table 3.—Reducing sugars and total sugars as mg/g fresh weight found in inbred lines of corn K44 susceptible and K41 resistant grown in the greenhouse and in the field[a].

	Greenhouse-grown, winter, young plants		Field-grown, summer, older plants	
	K44	K41	K44	K41
Reducing sugars	1.0	1.5	12.0	4.2
Total sugars, HCl	9.0	12.0	17.5	7.5
Total sugars, invertase	12.6	13.5	21.0	8.6

[a] Extracted with 80% ethanol.

Studies with nitrogen sources.—The basal medium contained 0.427 g/L of nitrogen supplied as 2 g/L of asparagine. When the amount of asparagine was varied from 0 to 4 g/L, growth of the fungus mycelium by the 7th day was greatest at the highest N concentration (Fig. 1-D). The yield of mycelium was about the same with 2 g/L and 4 g/L of asparagine, but increased progressively from no added asparagine to these highest concentrations. These results were essentially the same in two separate experiments.

To find out if differences in amino acids in the plant might be involved in resistance, qualitative determinations were made with filter paper chromatography (1) of the free amino acids in extracts of 3 susceptible and 7 resistant inbred lines. There was no consistent difference between susceptible and resistant lines for the amino acids detected by this method (Table 4).

Table 4.—Amino acids present in extracts of greenhouse-grown susceptible and resistant inbred lines of corn using qualitative paper chromatographic analysis.

Corn line	Disease reaction[a]	Aspartic	Glutamic	Alanine	Cystine	Serine	Glycine	Lysine	Asparagine	Glutamine	Unknown
K61	S	X	X	X	X	X	X		X	X	X
H7	S	X	X	X		X	X				X
K44	S	X	X	X		X	X	X	X		X
K41	R	X	X	X		X	X	X	X	X	
WF9	R	X	X	X		X			X	X	X
T8	R	X	X	X		X	X	X	X	X	X
J315	R	X	X	X		X	X	X	X		X
CI3	R	X	X	X		X	X	X	X		X
K4	R	X	X	X		X	X				X
38-11	R	X	X	X		X	X	X	X		X

[a] S = susceptible, R = resistant.

Table 5.—Weight of mycelium of *Helminthosporium carbonum* produced on the basal medium in which various sources of nitrogen were substituted at concentrations supplying equimolar amounts of nitrogen at the concentration supplied in the basal medium and with initial pH adjusted at pH 6.[a]

Nitrogen source	4 days		8 days		11 days	
	Dry wt. (mg)	Final pH	Dry wt. (mg)	Final pH	Dry wt. (mg)	Final pH
L-glutamic acid	38.9	6.8	99.1	8.3	84.6	7.9
L-aspartic acid	32.8	6.6	95.9	8.5	78.5	8.8
Casein hydrolysate (vitamin-free)	47.3	5.7	86.3	6.3	83.3	6.5
L-asparagine	27.9	5.8	74.0	6.9	67.8	6.8
L-glutamine	30.5	4.3	75.0	3.1	71.3	3.2
KNO_3	22.9	6.6	71.7	7.5	73.6	8.2
Ammonium tartrate	29.2	4.5	68.6	4.0	65.6	4.7
Aminoacetic acid (glycine)	27.1	4.6	59.1	3.7	61.3	2.6
Urea	30.8	5.5	60.1	6.9	55.2	7.1
No added N	5.4	5.8	2.6	5.9	6.3	5.9

[a] Data are averages of 2 flasks.

Glutamine sometimes showed up in extracts of K41, but not in those of K44. The extracts of resistant lines WF-9 and T8, and of susceptible line K61, also contained glutamine. An unknown substance consistently showed up in all but the K41 extracts. Of the amino acids that can be detected by the method employed, only a few were found.

Two separate but identical experiments were performed to determine the growth of *H. carbonum* on different sources of nitrogen. The results of one of the experiments are presented in Table 5. Since the chromatograms of free amino acids in the corn plants showed the presence of quantities of aspartic and glutamic acids, and glutamine was present in extracts of K41 but not K44, these three substances were included in the experiment. Reduced, nonreduced, and various forms of organic nitrogen were included. The data indicate that the fungus utilized all but atmospheric sources of nitrogen and that the amount of growth depended on the source of nitrogen (Table 5). Glutamic and aspartic acids resulted in the highest yield of mycelium, and glycine and urea in the lowest yields.

Relation of nitrogen source to pH.—The pH of the culture filtrate sometimes gives clues to the metabolism of the organism. On the basal medium, when initial pH was adjusted to 6.0, pH decreased the first few days after inoculation to pH 5 or lower, and later increased to pH 8 or higher on the 9th or 10th day. This change was a result of the utilization of the various sources of nitrogen, as seen in Table 5. The pH was lowest with glycine, which also gave next to the poorest growth. Except with urea, KNO_3, and glutamine, poorer growth was correlated with a decrease in the pH of the culture filtrate. Aspartic and glutamic acids caused pH to rise above 8. With glutamine, pH dropped to about 3, but with asparagine, differing from glutamine by one carbon atom in the chain, it rose to almost 7.0. When the initial pH of the basal medium was adjusted to various levels, however, an initial pH of 3.0 gave a greater growth of the fungus than the other values, and pH 8.0 depressed growth (Table 6). These differences disappeared on the 8th day of growth, when pH was about the same in all filtrates except the one with the highest initial pH.

Extracts of diseased and healthy plants.—No inhibition of growth of the pathogen by expressed sap of resistant plants could be demonstrated consistently, but when alcoholic extracts of diseased and healthy plants were incorporated into a solid medium, growth in diameter of the colony of the fungus was inhibited by the extract from diseased susceptible plants but not that from diseased resistant plants or from healthy resistant or susceptible plants (Table 7).

Discussion.—In this study of the nature of resistance to disease, the simplest approach seemed to be to express sap of susceptible and resistant plants, make this sap into a medium, and culture the fungus on the medium to determine whether an antifungal substance was present. This approach did not yield positive results. Ranker (13), however, found some slight reduction of growth of the corn smut fungus on leaf extracts of selfed resistant lines of corn. More recently, Virtanen et al (17) showed that there are antifungal agents in many plants and that the expressed juice inhibited the growth of fungus pathogens. In extracts of wheat and maize seedlings 2(3)-benzoxazolinone was subsequently shown to be the active antifungal agent. The growth of *H. carbonum* was inhibited by extracts of infected susceptible corn in direct contradiction to what was expected. The reduction in growth of the fungus on extracts of inoculated susceptible plants but not on extracts of inoculated resistant plants does not agree with the hypothesis of Kuć et al (7) that the cause of resistance is similar to that in nonhosts such as potato. According to their hypothesis the greatest amount of antifungal agent, if such it should be, should have been produced in the resistant plant as the direct result of infection. The fact that extracts of diseased susceptible plants inhibit growth of the fungus does not lend itself to any simple hypothesis. Host-parasite interaction may produce a substance that inhibits growth of the pathogen *in vitro*. Why this substance does not prevent growth of the pathogen in the susceptible plant is puzzling, the more so since extracts from diseased susceptible plants were inhibitory. Other explanations might involve a nutritional imbalance of perhaps key amino acids in the extracts, a substrate buffered to an unsuitable pH for maximum growth or the formation by the fungus of an end product which restricted growth.

Table 6.—PH at time of harvest and dry weight of *Helminthosporium carbonum* mycelium produced when the initial pH of the medium was adjusted to various pH values using 6*N* HCl and 6*N* NaOH.[a]

Initial	3 days		5 days		8 days	
pH	pH	(mg)	pH	(mg)	pH	(mg)
3.0	4.6	37.6	6.0	67.1		
4.3	5.2	15.4	5.8	44.7	7.8	69.3
5.1	5.3	13.5	6.0	46.6	7.8	68.3
5.8	5.6	14.2	6.1	43.7	7.9	66.5
8.0	7.0	11.7	7.1	28.6	8.4	66.0

[a] Data are averages of 2 flasks.

Table 7.—Linear growth rate of diameter of colony of *Helminthosporium carbonum* cultured on potato-dextrose agar plus alcoholic extracts of corn as indicated.

Type of medium	Diameter of colonies (mm)[a]			
	2 days	3 days	4 days	5 days
K41 healthy	23.5	43.0	74.0	84.5
K41 diseased	19.5	40.5	69.5	80.0
K44 healthy	20.0	38.5	65.5	73.5
K44 diseased	11.5	25.5	44.5	58.5
PDA control	25.0	43.0	68.0	76.5

[a] Average of 2 replicates.

Nitrogen metabolism may be involved in the resistant reaction even though free amino acid content was not correlated with resistance. Zscheile and Murray (18) found no correlation between free amino acid patterns and genetic differences controlling resistance in isogenic wheat plants. Benedict and Hildebrand (2), however, found the quantitative and qualitative change in amino acid composition of the growing host to be associated with host-parasite relations; but they concluded that even as exact a method as chromatographic analysis failed to demonstrate that the identifiable compounds were the ones used by the pathogen during infection. It is apparent from the data in Table 5 that *H. carbonum* uses some forms of organic nitrogen better than others. The higher yields of mycelium on glutamic and aspartic acids suggest that these compounds might be utilized as added carbon sources as well as sources of nitrogen. This might also be true for asparagine and could account for the increased yield as the asparagine concentration was increased (Fig. 1-D). Further studies are needed on the growth of the fungus with various amino acids and mixtures of amino acids as nitrogen sources.

The growth on glutamine and asparagine presents another interesting problem in that, though yields of mycelium are about the same on both sources, the medium containing glutamine dropped to about pH 3 whereas the medium containing asparagine invariably rose to pH 7, and sometimes 8. Glutamine and asparagine differ only in the number of carbon atoms in the molecule.

Differences in growth with different organic sources of nitrogen were obtained by Converse (4), who found that *H. gramineum* cultured on media containing various sources of amino nitrogen made it possible to classify the sources into 6 distinct groups on the basis of mycelial weights of the fungus. With *H. gramineum*, however, glycine ranked as a nitrogen source next to the group with the highest growth rate, whereas with *H. carbonum* glycine was one of the poorest sources of nitrogen.

None of the B vitamins induced marked changes in growth rate when compared to the basal medium or to the glucose-casein hydrolysate medium with no added vitamins. Of 3 *Helminthosporium* species studied by Elliot (5), only one, *H. avenae*, was found to be deficient, and it was partially deficient for thiamin only. Robbins and Kavanaugh (15) found no effect on *H. sativum* from adding a bios preparation from yeast. *Helminthosporium* spp. are apparently autophytic for vitamins.

The greatest effect of changes in the basal medium came from increasing the carbohydrate content. Small changes in this fraction of the medium or in plant extracts could easily mask any effects of other compounds that might be affecting growth. Differences in fungus growth on expressed sap of K41 and K44 could most likely be explained by differences in carbohydrate content.

It is obvious that this host-parasite relationship needs further clarification. Culture of the fungus on media containing various nitrogen sources, followed by testing for pathogenicity on the corn plants, might prove fruitful. Since strong saprophytic characteristics are evident, the hypothesis should be investigated that the fungus growth into cells was previously altered or killed by a toxin. Such a situation, demonstrated by Luke and Wheeler (11) and Krupka (6), is the subject of another report.—Department of Plant Pathology and Physiology, Virginia Agricultural Experiment Station, Blacksburg.

LITERATURE CITED

1. Awapara, J. 1958. Application of paper chromatography to the estimation of free amino acids in tissues. Arch. Biochem. Biophys. 19:172-173.
2. Benedict, W. A., and A. A. Hildebrand. 1958. The application of chromatographic methods to the study of the susceptibility of soybeans to stem canker. Can. J. Plant Sci. 38:155-163.
3. Cappellino, R. A., and C. M. Haenseler. 1958. Longevity of some graminicolous species of Helminthosporium under mineral oil. Phytopathology 48: 695-696.
4. Converse, R. H. 1953. The influence of nitrogenous compounds on the growth of Helminthosporium gramineum in culture. Mycologia 45:335-344.
5. Elliot, E. S. 1948. Effect of vitamins on growth of some graminicolous species of Helminthosporium and Fusarium. West Va. Univ. Bull. Proc. West Va. Acad. Sci. 20:65-68.
6. Krupka, L. R. 1959. Metabolism of oats susceptible to Helminthosporium victoriae and victorin. Phytopathology 49:587-594.
7. Kuć, J., A. J. Ullstrup, and F. W. Quackenbush. 1955. Production of fungistatic substances by plant tissue after inoculation. Science 122:1186-1187.
8. Levy, A. L., and D. Chung. 1953. Two dimensional chromatography of amino acids on buffered papers. Anal. Chem. 25:396-399.
9. Lilly, V. G., and H. L. Barnett. 1951. Physiology of the fungi. McGraw-Hill Book Co., Inc., New York.
10. Lilly, V. G., and H. L. Barnett. 1953. The utilization of sugars by fungi. West Va. Univ. Agr. Expt. Sta. Bull. 362T.
11. Luke, H. H., and H. E. Wheeler. 1955. Toxin production by Helminthosporium victoriae. Phytopathology 45:453-458.
12. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.
13. Ranker, E. R. 1930. The nature of smut resistance in certain selfed lines of corn as indicated by filtration studies. J. Agr. Research 41:613-619.
14. Ranker, E. R. 1930. Apparatus and methods for obtaining sterile filtrates of biological fluids. Phytopathology 20:569-573.
15. Robbins, W. J., and V. Kavanaugh. 1942. Vitamin deficiencies of the filamentous fungi. Botan. Rev. 8:411-471.
16. Ullstrup, A. J. 1952. Leaf blights of corn. Indiana Agr. Expt. Sta. Bull. 572.
17. Virtanen, A. I., P. K. Hieltala, and O. Wahlroos. 1957. Antimicrobial substances in cereals and fodder plants. Arch. Biochem. Biophys. 69:486-500.
18. Zscheile, F. P., and H. C. Murray. 1957. Chromatographic study of amino acid development in wheat ovules in relation to genes for resistance. Phytopathology 47:631-632.

INFECTION OF HOP CROWNS AND ROOTS BY PSEUDOPERONOSPORA HUMULI AND ITS RELATION TO CROWN AND ROOT ROT AND OVERWINTERING OF THE PATHOGEN

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SUMMARY

Hop downy mildew systemically infected all subterranean portions of the hop plant, including roots, rhizomes, and crowns. Many of the infected crowns died but others lived for a number of years.

Symptoms of mildew on the belowground parts include a reddish-brown discoloration and a brown necrotic spotting in the parenchyma tissue of the cortex, xylem, phloem, and pith. These symptoms are often accompanied by a black rot assumed to be due to secondary organisms. Mycelium and haustoria of *Pseudoperonospora humuli* were found in the phloem, cortex, pith, and parenchyma cells of the xylem. The mildew overwinters in the crown and invades new shoots, causing basal spikes on which the mildew sporulates. Secondary spread occurs from these shoots by means of sporangia. When shoots become infected, the fungus spreads systemically into the crown, completing the life cycle. Oospores, found only once, are considered unimportant in the life history of the hop downy mildew fungus in the Yakima Valley in Washington.

The hop downy mildew fungus, *Pseudoperonospora humuli* (Miy. & Tak.) G. W. Wils., was reported on hops, *Humulus lupulus* L., in western Washington in 1930 (4). When it appeared in eastern Washington is not definitely known, but it had not yet been found there as late as 1933 (5).

Hop downy mildew, like most downy mildews, has been recognized mainly as infecting the aboveground portions of the hop plant. It causes a cone blight, shoot stunting, and local lesions on the leaves (5). The last two symptoms have been observed in the hop-growing area of the Yakima Valley every year, though cone blight is rare. In England, Ware (10) found this fungus overwintering as perennial mycelium in the hop crown. Other workers (1,7) have concluded that such crown infections are unimportant in their areas in the life history of this pathogen.

In recent years a serious crown and root die-out has occurred in the Yakima Valley hop yards. The results of investigations to determine the role of the hop downy mildew fungus in this die-out and phases of its life history are reported herein.

Histology.—Freehand sections, cut with a razor blade, were stained with cotton blue in lactophenol and gently heated; excess stain was drained off and the sections were mounted in clear lactophenol.

Haustoria and mycelium characteristics of *P. humuli* could be readily discerned. They were similar to those found in infected shoots and also similar to those described by Millasseau (8). The haustoria were most useful in diagnosing mildew infections. The haustoria were centered in groups of cells rather than uniformly distributed throughout the tissue of the infected area. Haustoria were observed in cortex, phloem, pith, the parenchyma cells of the xylem, and crowns, rhizomes, and roots 12–18 in. deep. These observations are in agreement with those of Ware (10), who traced systemic infections into the hop root system.

Symptoms.—The mildew infection in hop roots, rhizomes, and crowns is centered in the pith, phloem, xylem parenchyma, and cortex. One of the most common symptoms is a reddish-brown discoloration of infected tissue (Fig. 1-A1,B2). The cut surface of this tissue has a moist appearance. This symptom is apparently associated with young infections. The older diseased tissue contains dark brown necrotic spots from one to several millimeters in size, sometimes appearing as streaks (Fig. 1-C).

Quite often a black rot is found in combination with the two symptoms described (Fig. 1-B3,4). This black rot is usually sharply delimited from infected but unrotted tissue. It is usually found on roots and rhizomes that are composed largely of parenchyma cells. A similar black rot has also been associated with infections by *Gibberella pulicaris* (Fr.) Sacc. var. *minor* Wr. (3) and *Phytophthora cactorum* (Leb. and Cohn) Schroet. (2) as well as on hops grown in heptachlor-treated soil (6).

Life History.—The first evidence of hop downy mildew is the systemically infected hop shoots (basal spikes) that grow from diseased crowns, beginning to appear in late April (Fig. 1-E1). The internodes of these shoots are shorter than normal shoots (Fig. 1-E3). Shoots stunted by secondary infections can usually be distinguished from the basal spikes by the presence of one or more normally elongated internodes at the base (Fig. 1-E2). Shoots such as these have rarely been associated with systemic infeciton.

A field experiment was conducted in 1959 to determine how readily the mildew could infect the crown of the Early Cluster variety by way of infected shoots. Shoots of various lengths were inoculated in the evening of May 2 with sporangia and covered overnight with plastic bags to ensure high moisture conditions. This inoculation resulted in anywhere from 2 to over 20 diseased shoots on each crown. These shoots were growing from one-year-old hop crowns that were free of mildew infection. In another test,

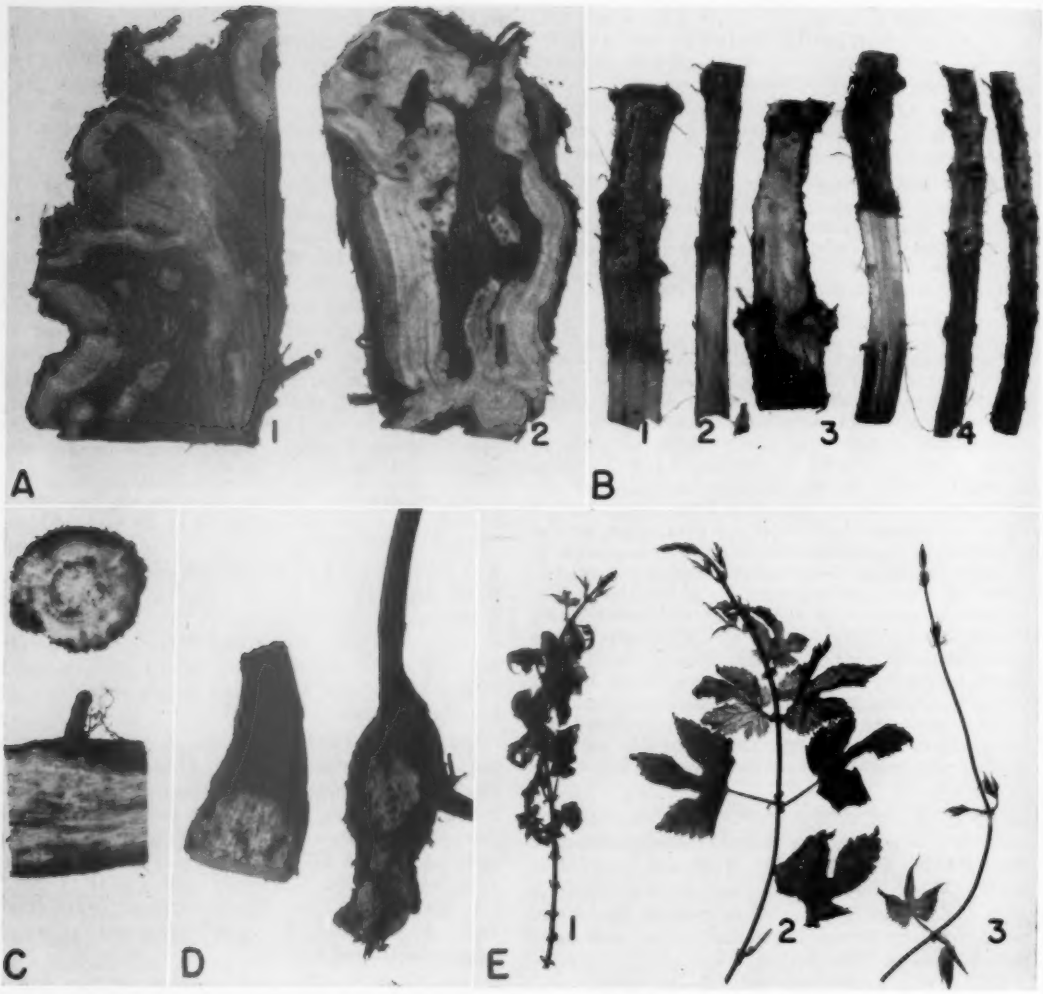

Fig. 1. **A)** Longitudinal section of a hop crown showing: 1) infected and 2) healthy crown. **B)** Hop rhizomes: 1) healthy, 2) reddish-brown discoloration, 3, 4) black rot symptoms. **C)** Hop root showing dry brown spotting symptom. **D)** Base of a downy mildew infected bine (note swelling). **E)** Hop shoots: 1) basal spike systemically infected from crown, 2) typical secondary infected shoots (similar shoots rarely occur from systemic crown infection), 3) healthy shoot.

the lower nodes of trained bines were inoculated after the basal leaves of the bine and excess shoots were removed from the hop crown. These bines were not stunted, and the only evidence that they were infected were the diseased lateral branches that grew out after inoculation. Uninoculated checks were included.

During the summer, 3% of the bines that had shoots of various lengths had died. Four per cent of the bines in which the lower nodes of the bine were inoculated, died. These bines that died had grown well and generally could not be distinguished from healthy bines. Tissue from the lower nodes of these dead bines was examined, and all were infected with mildew. Bines that had died generally had an enlarged base (Fig. 1-D) that tapered to a small diameter at the point of attachment to the crown. Actual death of the bine was generally due to its breaking off at the point of attachment to the crown. No bines died in the uninoculated plots.

In December the crowns were uncovered and examined for mildew infection (Table 1). *P. humuli* readily infected the hop crown by spreading down through the inoculated shoots. A number of crowns whose shoots were not inoculated became infected by natural secondary spread from the shoots originally inoculated. Mildew infection in new rhizomes was

TABLE 1. Infection of hop crowns with hop downy mildew by the inoculation of shoots

Shoot length when inoculated	Inoculated shoots		Uninoculated shoots	
	No. crowns infected	% crowns infected	No. crowns infected	% crowns infected
1–4 in.	40	70	8	19
1–3 ft[a]	23	70	3	16
Base of bine[b]	6	24	2	11

[a] Shoots inoculated at tips.
[b] Side branches, leaves, and small shoots were removed and the lower nodes of trained bines were inoculated.

general. An examination of rhizomes offered for sale for planting of new hop yards showed as many as 10% with obvious mildew infections.

In June of 1960, 71 of the crowns of the inoculated plants were again uncovered and examined. Of the crowns infected in December, 75% were dead in June. Thirty-eight per cent of the crowns whose shoots had been inoculated but not found infected earlier, were also dead. Shoots grown from the surviving crowns were healthy, and only one infected basal spike was found. The uninoculated checks were not re-examined.

The results show that the hop mildew can readily infect hop crowns, at least the Early Cluster variety, by mycelial growth downward in infected shoots. That is emphasized by the number of uninoculated crowns that became infected by secondary spread. Infected rhizomes used as planting stock introduce the disease in new plantings. Production of basal spikes from infected crowns is relatively rare, but frequent enough to initiate secondary spread throughout the hop yard. The mildew infection is implicated as an important factor in the crown die-out by the death in the first winter of 75% of the infected crowns found infected in December and 38% of the inoculated crowns that seemed healthy in December.

The natural development of hop downy mildew was followed for 4 years in a field plot containing 368 crowns (about 2/5 acre). This hop yard was several years old. In April, 1957, basal spikes were growing from 14% of the crowns. All crowns that produced basal spikes were infected with mildew; no others were examined. In 1958 basal spikes were found on 18% of the crowns, and all of these crowns were infected. Four per cent of these crowns had produced basal spikes in the previous year, too. The remainder of the crowns infected in 1957 showed no basal spikes but were still infected or had died. After two years, 22% of the crowns in this plot were infected with hop downy mildew.

In 1959, all 368 crowns were examined for mildew infection and 40% were infected. In 1959 6% of the crowns produced basal spikes, of which 2% had basal spikes in 1958 and 1% had basal spikes in both 1957 and 1959. Only 2 crowns produced spikes in all 3 years.

In 1960, all of the crowns were examined again, and 57% were infected with mildew. The weather was favorable for mildew development in 1959, undoubtedly accounting for the increased number of infected crowns in the spring of 1960. Only 2 crowns that had not shown diseased shoots in previous years showed them in 1960. One crown produced basal spikes in 1959 and again in 1960, one had spikes in 1958 and 1960, and one had spikes in 1957, 1959, and 1960. None produced basal spikes in all four years. Many of the crowns infected in previous years were dead and had been replanted. In other hills new plants had grown from rhizomes that had grown from the original crown. It is interesting to note that only a small number of infected crowns produced basal spikes year after year.

Oospores of hop downy mildew have been reported to occur in infected leaf, petiole, cone, and stem tissue (1). During the past four growing seasons a search was made for oospores. Infected leaf, petiole, and stem tissue was collected periodically throughout the growing season. The chlorophyll was removed with acid-alcohol, and the tissue was cleared with carbol-xylol and stained with cotton blue in lactophenol. Oospores were found in only one instance—in a basal spike collected in May, 1957. Thus, though oospores do occur in the Yakima Valley, they appear to be less common than in other areas, and of little importance in the overwintering of hop downy mildew in that area.

DISCUSSION.—The hop downy mildew fungus, *P. humuli*, has been responsible for the migration of the hop industry to arid regions in the Pacific Northwest. In central Washington, particularly in the Yakima Valley, rainfall over a 33-year period averaged less than 2½ in. from April to September (9). This climate reduces the incidence of leaf and cone infections, but has not prevented the occurrence of basal spikes and crown infection. The ability of *P. humuli* to exist under the arid conditions of Washington indicates the infectious nature of this pathogen.

Infected crowns that produce infected basal spikes are the source of primary infection, and essential for continued existence of the pathogen. These infected shoots occur every spring. All that is necessary are suitable conditions for sporulation and the presence of moisture so the sporangia can germinate and infect other shoots. These conditions have occurred in 3 of the past 4 growing seasons, and fungus has been able to infect new crowns by advancing downward through the infected shoots. Many of these crowns die by the following spring; some persist for 3–4 years; others die but a rhizome becomes established and sends up healthy shoots.

This work confirms that of the English workers. However, Arens (1), working in Germany, and Magie (7), in New York, concluded that oospores were the means of overwintering in their respective areas. In Washington there can be no doubt that the main means of overwintering of the hop downy mildew is infected crowns that produce infected shoots from which secondary spread occurs.

The importance of oospores is not certain, but a hop yard that had severe die-out incited by mildew was replanted in 1957 and the disease has not been found in this yard to date. If oospores were an important factor in overwintering, one would expect that this yard would have many infected crowns by now. In addition, hundreds of basal spikes have been traced to point of attachment to the parent plant, and in every instance but one they were found to be growing from crowns or rhizomes extensively infected by hop mildew.

LITERATURE CITED

1. Arens, K. 1929. Untersuchungen über Pseudoperonospora humuli (Miyabe u. Takah.) den Erreger der neuen Hopfenkrankheit. Phytopathol. Z. 1:169-193.
2. Christie, T. 1956. Black root rot, Phytophthora cactorum (Leb. and Cohn) Schroet, of the hop plant. New Zealand J. Sci. Tech. Sec. A 38:277-284.
3. Christie, T. 1957. A black rot of the hop plant. New Zealand J. Sci. Tech. Sec. A 38:647-648.
4. Heald, F. D. *In* Fortieth annual report for fiscal year ended June 30, 1930. Washington Agr. Expt. Sta. Bull. 245, p. 49.
5. Hoerner, G. R. 1933. Downy mildew of hops. Oregon State Agr. Coll. Ext. Bull. 466, 8 p.
6. Klostermeyer, E. C., and C. B. Skotland. 1959. Pesticide chemicals as a factor in hop die-out. Washington Agr. Expt. Sta. Circ. 362, 11 p.
7. Magie, R. O. 1942. The epidemiology and control of downy mildew on hops. New York State Agr. Expt. Sta. (Geneva) Tech. Bull. 267, 48 p.
8. Millasseau, J. 1929. Contribution à l'étude morphologique du Peronoplasmopora humuli Miya. et Tak. Ann. des epiphytes 14:177-198.
9. Nelson, C. E., and C. E. Domingo. 1958. Data on weather from 1924-1957 at the Irrigation Experiment Station near Prosser, Wash. Wash. Agr. Expt. Sta. Bull. 593, p. 22.
10. Ware, W. M. 1926. Pseudoperonospora humuli and its mycelial invasion of the host plant. Brit. Mycol. Soc. Trans. 11:91-107.

BIOCHEMICAL CHANGES IN SHELLED PEANUTS CAUSED BY STORAGE FUNGI I. EFFECTS OF ASPERGILLUS TAMARII, FOUR SPECIES OF A. GLAUCUS GROUP, AND PENICILLIUM CITRINUM

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SUMMARY

Aspergillus tamarii, 4 species of the *A. glaucus* group, and *Penicillium citrinum*, fungi previously isolated from stored peanuts, were grown on autoclaved shelled peanuts. At intervals of 2, 4, and 8 weeks, organic-matter loss and water formation were determined, and chemical analyses made of oil, sugars, and protein. The principal biochemical changes were loss in organic matter, a complete degradation of sucrose, a decrease in total oil, an increase in free fatty acids, and an increase in per cent of unsaturated fatty acids of the oil. Under the optimum moisture conditions of this experiment, *A. tamarii* caused the greatest changes, the *A. glaucus* group was intermediate, and *P. citrinum* caused the smallest changes. The fungi did not effect peroxide values, total carbonyls, total tocopherols, or protein nitrogen. Thus the main type of deterioration was a hydrolytic rancidity of the oil. This resulted in a dark reddish-orange-colored oil with moldy-acrid odor. No evidence indicated an oxidative type of rancidity.

Peanuts stored at varying kernel moistures under controlled laboratory conditions showed increases in respiration and free fatty acids with time at moisture contents of 7% or higher (23). The number of fungi associated with stored peanut kernels was later related to initial moisture content (5). These studies suggested that fungi may be the cause of the deterioration of peanuts stored at moisture contents of 7% and above. Christensen (3) and Semeniuk (21) recently reviewed seed microbiological research that related fungi to deterioration in stored corn, soybean, and wheat seeds. Milner and Geddes (17) reviewed respiratory and biochemical changes resulting from the invasion of stored seed by fungi. Altschul (1) attributed deterioration in cottonseed to the biochemical activity of the living cells of the seed.

Most studies on biochemical changes in stored seeds have been with fungus-infested samples taken at various intervals of time from small lots of seeds stored at different moisture contents under controlled laboratory conditions. In some cases, samples were taken from commercial-type bulk storage. Oil seeds so studied were soybean and cotton. Soybean seed showed decreases in nonreducing sugars and iodine number, increases in reducing sugars and free fatty acids, and little change in total oil and nonprotein nitrogen (15). Deteriorated cottonseed showed decreases in total sugars, increases in free fatty acid, and protein hydrolysis (1).

Many different species of fungi have been found in stored seeds. In only a few instances, however, has the role of the individual fungus in causing biochemical

changes been determined. Nagel and Semeniuk (20) inoculated autoclaved shelled corn with various species of *Aspergillus* and *Penicillium*, and evaluated each species as to its effect on fat acidity, organic matter, water-soluble nitrogen, and pH. Milner and Geddes (16) and Goodman and Christensen (8) used the same method to assay the lipolytic activity of storage fungi on soybeans and corn, respectively.

This paper reports quantitative biochemical changes in autoclaved shelled peanuts caused by pure cultures of storage fungi described in a previous report (5).

MATERIALS AND METHODS.—Whole shelled peanuts (*Arachis hypogaea* L. var. Dixie Runner) that failed to pass a screen of 15/64×¾ in. mesh served as the substrate for the following fungi: *Aspergillus chevalieri* (*Eurotium chevalieri* Mangin), *A. repens* (*E. repens* d B.), *A. restrictus* G. Smith, *A. ruber* (*E. rubrum* Brewer), *A. tamarii* Kita, and *Penicillium citrinum* Thom.

Peanuts selected for uniformity and with a moisture content of 6.75% were shelled, and 150 g were placed in 500-ml Erlenmeyer flasks. The flasks were plugged with cotton and autoclaved 30 minutes at 15 lb pressure. Twenty-five ml of hot, sterile demineralized water were added to each flask to bring seed moisture to about 20% moisture (wet-weight basis). The flasks were shaken by hand 3 times a day for 3 days, after which period all visible moisture had been absorbed. Seven flasks were then inoculated with a loop of spores from 10-day-old test-tube cultures of each of the 6 fungi. The remaining flasks were left as controls. For the initial control samples, flasks were randomly selected from the group before and after autoclaving. All flasks were incubated at 30°C. Two flasks of each treatment and the control were removed at 2, 4, and 8 weeks after inoculation. These samples were weighed, placed in wire baskets, and dried for 72-96 hours under 2 infrared lights (250-watt) at a distance of 75 cm. The fungus in the samples was then killed by 20 minutes in a forced-draft oven at 130°C, and the seed was reweighed. After grinding in a food chopper with a 16-point blade, a 25-g sample was placed in a weighing can for 5 hr in a forced-draft oven at 135°C to determine remaining moisture. Final dry weight was determined by subtracting the weight of the remaining moisture from the weight of the peanuts after drying at 130°C for 20 minutes. Initial dry weight was 140 g. The ground samples were stored in screw-cap jars at 4°C until chemical analyses were made.

Loss of organic matter was determined by subtracting the final dry weights of the fungus-inoculated peanuts at 2, 4, and 8 weeks from that of the control. At 2, 4, and 8 weeks the amount of water in the control and the fungus-inoculated peanuts was calculated. The amount of water in the control was subtracted from the amount of water in the fungus-inoculated peanuts to give an estimate of the water formed by the metabolism of the fungi.

Total oil was determined on duplicate 2-g samples by extraction for 12 hours (Soxhlet) with petroleum ether (b.p. 30-60°C) by AOCS Method Ab 3-49 (2). After petroleum-ether extraction, the residue was extracted (Soxhlet) with 70% ethyl alcohol to remove the sugars for analysis. The ethyl-alcohol extract was evaporated on a steam bath to about 10 ml, cleared, and analyzed for reducing and non-reducing sugars by the Hassid method as outlined by Joslyn (12). Duplicate 30-mg samples from the alcohol-extracted residue were analyzed for protein nitrogen by the micro-kjeldahl method of Kirk (13).

Free fatty acids were determined on duplicate 7.05-g samples of oil by AOCS Method Ab 5-49. Free fatty acids were calculated as per cent oleic acid. Iodine number was determined in triplicate by the Wijs 30-minute procedure outlined in AOCS Method CD 1-25. Two measurements of the percentage of linoleic acid were made by the ultraviolet spectrophotometric method of polyunsaturated acids given in AOCS Method CD 7-48. The percentages of linoleic, oleic, and saturated acids in the sample were calculated by the formulas:

$$\% \text{ linoleic acid} = 1.086 \frac{(\text{O.D. at } 233 \text{ m}\mu)}{0.04}$$

$$\% \text{ oleic acid} = \frac{\text{iodine number} - (1.811 \times \% \text{ linoleic acid})}{0.899}$$

% saturated acids = 100%−% linoleic acid−% oleic acid.

Peroxide values were determined on duplicate 1-g samples of oil by the Wheeler method as modified by Moore and Bickford (18). Total carbonyls were determined in duplicate on benzene extracts of 0.5 g of peanuts by the 2,4-dinitrophenyl hydrazine method of Henick et al (9). Total tocopherols were analyzed in duplicate 500-m samples of peanut oil after dissolving in n-hexane by the method of Hove and Hove (11).

Oil color was estimated visually.

RESULTS.—*Evaluation of heat treatment.*—The effect of steam sterilization was evaluated by comparing the analyses of the chemical constituents of autoclaved peanuts with those of raw peanuts of the same lot. It was recognized that autoclaving caused other physical and chemical changes in the peanut kernel substrate, but the only chemical tests in this phase of the investigation were those reported for inoculated peanuts. No significant changes were found in per cent of oil, nitrogen fraction, fatty acid composition, tocopherols, or iodine value. Peroxide number, free fatty acids, and reducing and total sugars were reduced 20–25%, and carbonyls about 40%. The results of chemical analyses reported subsequently are for inoculated peanuts compared to controls of uninoculated autoclaved peanuts held for the same length of time under identical laboratory conditions.

Loss of organic matter.—Each of the 6 species of fungi caused a loss of organic matter in shelled peanuts at intervals of 2, 4, and 8 weeks (Table 1). Differences were only small within the *A. glaucus*

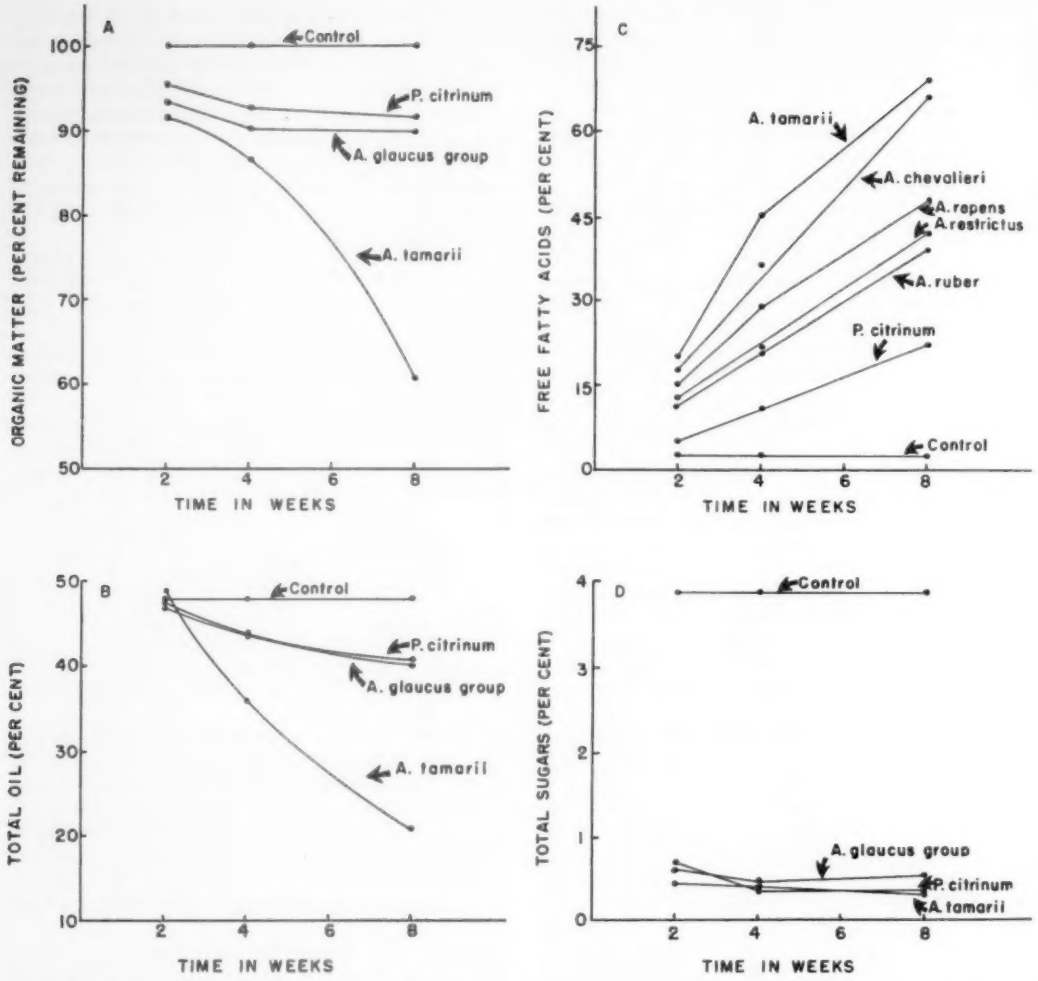

Fig. 1. Changes in chemical composition of fungus-inoculated peanuts at 2-, 4-, and 8-week intervals. **A)** Organic matter as per cent remaining of initial dry weight. **B)** Total oil as per cent of initial dry weight. **C)** Free fatty acids expressed as per cent oleic acid. **D)** Total sugars as per cent of initial dry weight.

group (*A. repens, A. restrictus, A. chevalieri,* and *A. ruber*). Consequently, when the organic matter loss data were expressed on a per cent remaining basis, the 4 species of *A. glaucus* group were averaged (Fig. 1-A). The greatest loss in organic matter was caused by *A. tamarii* (39%), with the *A. glaucus* group intermediate (10%), and *P. citrinum* causing only a small loss (8.2%). Growth of *A. tamarii* resulted in a constant rate of organic matter loss in peanuts during the entire 8 weeks. The *A. glaucus* group and *P. citrinum* caused a much higher rate of loss for the 2- to 4-week interval than for the 4- to 8-week interval.

Changes in oil fraction.—After 2 weeks of growth, the fungi had not appreciably affected the total oil, but after 4 and 8 weeks of growth, decreases had occurred (Fig. 1-B). *A. tamarii* caused a more rapid decline and a greater loss of oil than did the *A. glaucus* group and *P. citrinum*.

Each of the 6 species of fungi caused an increase in free fatty acids of the oil (Fig. 1-C). This increase continued with time and at an almost constant rate that differed with each species of fungus. After 8 weeks, growth of *A. tamarii* and *A. chevalieri* had resulted in the highest percentages of free fatty acids, respectively 69 and 66%. Growth of the other 4

TABLE 1. Effect of *Aspergillus* and *Penicillium* fungi on water formed, organic matter loss, and ratio of water formed to organic matter loss (WF/OML) of peanuts at 2-, 4-, and 8-week intervals (initial dry O.M.—140 g)

Fungus	Week	Water formed (g)	Organic matter loss (g)	WF/OML (ratio)
A. tamarii	2	11.2	10.4	1.08
	4	24.7	19.4	1.27
	8	30.3	39.1	0.77
A. repens	2	10.0	8.7	1.14
	4	10.1	14.8	0.68
	8	17.0	16.9	1.00
A. restrictus	2	8.5	7.5	1.13
	4	10.1	11.8	0.86
	8	17.4	13.1	1.31
A. chevalieri	2	8.2	8.5	0.97
	4	17.8	12.6	1.41
	8	16.9	13.2	1.26
A. ruber	2	8.5	8.5	1.00
	4	9.4	12.8	0.74
	8	9.0	13.1	0.69
P. citrinum	2	4.4	6.0	1.34
	4	6.3	10.5	0.60
	8	6.8	11.5	0.59

TABLE 2. Effect of *Aspergillus* and *Penicillium* fungi on fatty acid composition of peanut oil at 2-, 4-, and 8-week intervals

Fungus	Week	Fatty acid composition (%) Oleic acid	Linoleic acid	Saturated acids
None (control)		58.8	17.5	23.7
A. tamarii	2	54.8	19.2	26.0
	4	62.5	15.5	22.0
	8	63.2	19.4	17.4
A. ruber	2	59.6	16.9	23.5
	4	58.1	18.6	23.3
	8	57.8	19.2	23.0
A. chevalieri	2	57.8	19.2	23.0
	4	62.5	14.4	23.1
	8	72.8	13.9	13.3
A. repens	2	55.9	12.1	27.0
	4	60.6	19.4	20.0
	8	65.8	14.1	20.1
A. restrictus	2	58.7	17.9	23.4
	4	50.2	21.1	28.7
	8	66.5	17.4	16.1
P. citrinum	2	65.4	14.8	19.8
	4	58.7	17.2	24.1
	8	68.6	15.4	16.0

species of fungi resulted in percentages ranging from 47% to 21%.

Growth of the fungi on shelled peanuts did not result in appreciable increases in peroxide value, total carbonyls, or iodine number of the oil. Total tocopherols varied some, but apparently not from fungal growth, since no pronounced trend toward loss was noted.

Table 2 shows the effect of the 6 species of fungi on the fatty-acid composition of the oil. Each fungus caused a decrease of about 6–10% in saturated acids, with a corresponding increase in unsaturated acids of the peanut oil. Except with *A. ruber*, oleic acid accounted for the principal increase in the unsaturated acids.

In addition to the biochemical changes in peanut oil, all of the species of fungi except *P. citrinum* caused a marked change in color of the oil (Table 3). Depending on time and species, the peanut oil was yellow, orange, or amber. The fungi appeared to synthesize two kinds of pigments, one soluble in petroleum ether and the other soluble in 70% ethyl alcohol. The fungi also changed the organoleptic properties of the oil (Table 3). The *Aspergillus* species caused a change from the typical nutty odor of peanut oil to oils that had moldy, acrid-burnt, or sweet-burnt odors. *P. citrinum* produced an oil without any particular odor. None of the fungi caused a rancid odor generally associated with oxidative rancidity. This was not surprising, since the growth of the fungi did not increase peroxide values and total carbonyls.

Changes in sugars and protein nitrogen.—Autoclaved peanuts contained 3.87% total sugars (3.35% sucrose and 0.52% reducing sugar). The data (Fig. 1-D) are plotted as per cent total sugars, but from the low percentage of sugars remaining at the 2-week interval it is obvious that the fungi had depleted the sucrose fraction. Even so, some reducing sugar remained at the 4- and 8-week intervals.

Growth of the fungi at the 2- and 4-week intervals generally caused a reduction of less than 0.5% in protein nitrogen (Fig. 2-A). At the 8-week interval, however, except with *A. tamarii*, the protein nitrogen in the fungus-inoculated peanuts was not appreciably different from that of the control. Since

TABLE 3. Color of petroleum ether and 70% ethyl alcohol extracts from peanuts inoculated with *Aspergillus* and *Penicillium* fungi (with an evaluation of odor of oil)

Fungus	Petrol ether 4 wk	Petrol ether 8 wk	Ethyl alcohol 4 wk	Ethyl alcohol 8 wk	Odor of oil
None (control)	None	None	None	None	Nutty
A. tamarii	Yellow	Orange	Yellow	Yellow	Acrid-burnt
A. ruber	Orange	Orange	Amber	Amber	Moldy
A. chevalieri	Yellow	Orange	Yellow	Amber	Moldy
A. repens	Yellow	Orange	Amber	Amber	Sweet-burnt
A. restrictus	Yellow	Yellow	Yellow	Yellow	Sweet-burnt
P. citrinum	None	None	None	None	Flat

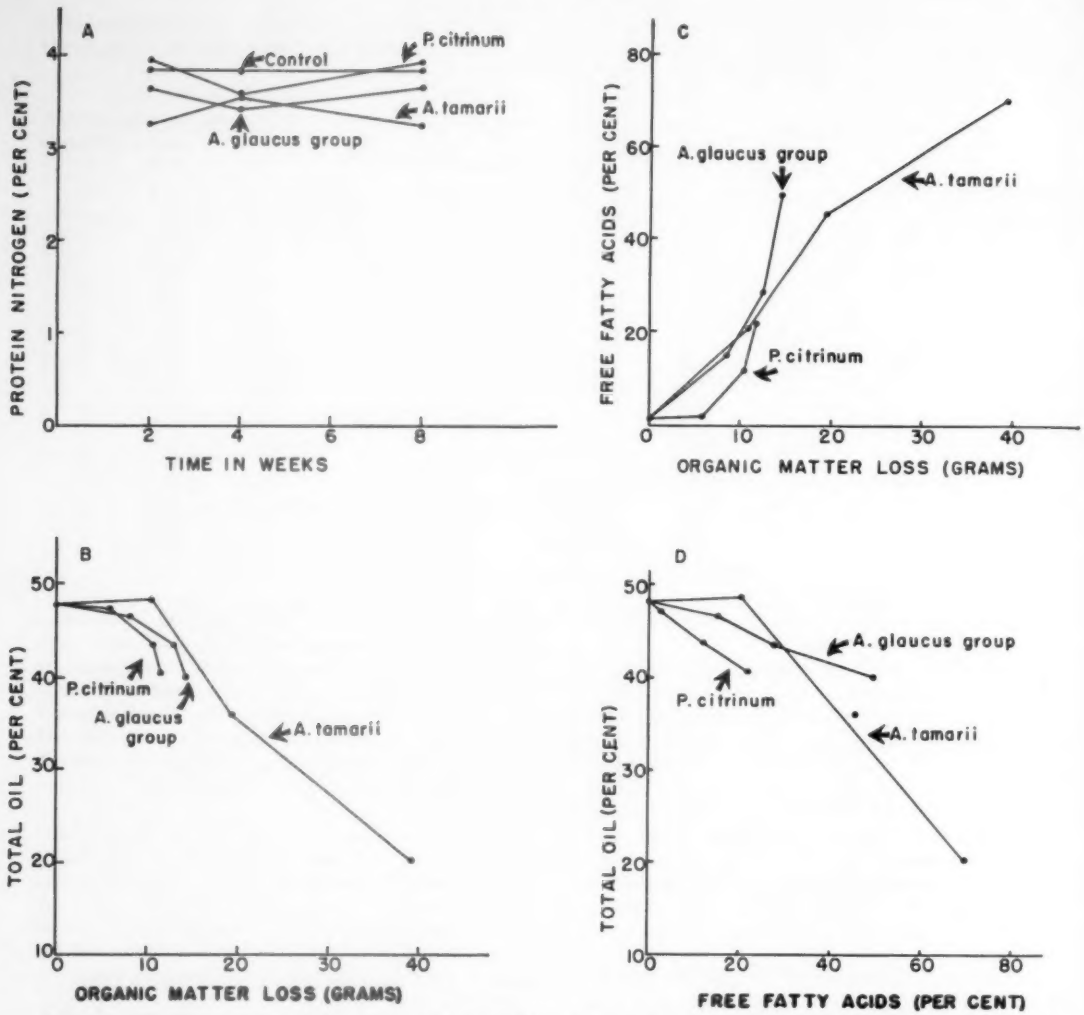

Fig. 2. Changes and relationships in chemical composition of fungus-inoculated peanuts at 2-, 4-, and 8-week intervals. **A)** Protein nitrogen as per cent of initial dry weight. **B)** Total oil in relation to organic matter loss as per cent of initial dry weight and as grams, respectively. **C)** Free fatty acids in relation to organic matter loss as per cent oleic acids and as grams, respectively. **D)** Total oil in relation to free fatty acids as per cent of initial dry weight and as per cent oleic acid, respectively.

there were no large decreases in protein nitrogen, the growth of these fungi probably caused no large increases in soluble nitrogen.

Organic matter losses in relation to water formed, total oil, and free fatty acids.—The ratio between organic matter loss and water formed is more than 1, as brought out by the actual ratio figures in Table 1. On complete oxidation 1 g of oil yields 1.07 g of water. The ratio obtained here indicated that the fungi were utilizing the oil. The ratios of less than 1 at the 8-week period suggested that the fungi may have utilized both the oil and another substrate.

Fig. 2-B shows the relation between organic matter loss and total oil at 2-, 4-, and 8-week intervals. At the 2-week interval, the amount of organic matter had decreased more rapidly than total oil. This indicated that sugars were the source of organic matter loss during the first 2 weeks of growth. At the 4- and 8-week intervals, the quantity of organic matter loss and the decrease in oil were more nearly proportional. Thus, during the 4- and 8-week periods, organic matter loss was the result of utilization of oil by the fungi.

Organic matter loss was obviously related to fatty-acid production for the 2- and 4-week periods for all of the fungi (Fig. 2-C). This relationship continued for 8 weeks with *A. tamarii*, but not with the *A. glaucus* group and *P. citrinum*. These produced free

fatty acids even after organic matter losses had practically ceased.

Total oil in relation to free fatty acids.—Fig. 2-D shows that the *A. glaucus* group and *P. citrinum* caused a more rapid increase in free fatty acids than decrease in total oil. For *A. tamarii* the situation was similar at the 2-week interval, but the rates were about equal at the 4- and 8-week intervals. The relationship of total oil to free fatty acids was very similar to that of organic matter loss to free fatty acids (Fig. 2-C).

Discussion.—Since no method had been developed to obtain sterile viable peanut seed, it was decided that data on the biochemical changes induced by pure cultures of fungi grown on autoclaved peanuts would be more valuable than data from changes occurring in mixed cultures. The species in mixed cultures vary with seed source. Antagonism and preincubation (curing and storage) environment might also be important in the makeup of the microflora causing biochemical changes. The authors realized the limits of this method for drawing implications on the deterioration of peanuts in storage. Nevertheless, they also hoped to evaluate the potentiality of the individual species of fungi to cause biochemical changes in peanuts under these experimental conditions.

The major biochemical changes in peanuts from the growth of storage fungi under these experimental moisture and temperature conditions were a loss of organic matter, a complete loss of sucrose, a decrease in oil, and an increase in free fatty acids. A minor change was an increase in per cent of unsaturated fatty acids in the oil. There were no appreciable changes in peroxide values, total carbonyls, and total tocopherols. The high free-fatty-acid percentages showed that the fungi had caused a hydrolytic type of deterioration in peanut oil. Associated with this hydrolysis was a discoloration of the oil plus an acrid-moldy odor.

All species of fungi completely utilized sucrose after 2 weeks of growth. This probably accounted for the major loss in organic matter for the first 2 weeks. The ratio greater than 1.0 of organic matter loss to water formed suggested oxidation of the oil fraction, but percentage total oil did not decrease during the 2-week interval. A possible explanation is that the fungi synthesized a part of the sucrose into oil.

The loss in organic matter, formation of water, and increase in free fatty acids in fungus-inoculated peanuts are in agreement with the findings of Nagel and Semeniuk (20) with different species of *Aspergillus* and *Penicillium* grown on autoclaved corn. The results reported here, however, showed which constituents of the peanut were the source for organic matter loss at the various periods. Although Nagel and Semeniuk found that growth of fungi on corn decreased protein nitrogen, growth of fungi on peanuts did not affect protein nitrogen appreciably, which agrees with the data of Milner (15) on soybeans.

The production of free fatty acids by *A. tamarii*, the *A. glaucus* group, and *P. citrinum* in this study is in agreement with similar studies with other species of *Aspergillus* and *Penicillum* on corn (8) and soybeans (16). The utilization of the sucrose fraction in peanuts by fungi is similar to that observed on soybeans (16). A major difference, however, was an accumulation of reducing sugars in soybeans but not in peanuts.

Milner (15) showed that the iodine number was lower in oil from fungus-infested soybeans than in oil from mold-free soybeans. Fungus growth on peanuts, however, did not affect iodine number, but was associated with an increase in the per cent of unsaturated fatty acids of the peanut oil, which was mainly due to an increase in per cent oleic acid. Foster (7) and Cochrane (4) reported that the principal kind of fatty acid in the mycelium of species of *Aspergillus* was oleic acid.

Since peroxide values and total carbonyls were not appreciably affected by fungus growth, it is concluded that the species of fungi studied here did not induce autoxidation of the oil. This is to be expected since the natural antioxidant, the tocopherols, was not destroyed by fungi nor was there an increase in linoleic acid. Thus the fungi did not change the fatty-acid composition of the peanut oil sufficiently to induce autoxidation. Holman (10) and Morris (19) pointed out that autoxidation usually occurs when the oil either has a high linoleic acid content or has a reduction in tocopherols. The tocopherol values reported here are well within the reported range of 0.26–0.59 mg per g of peanut oil (14). Fore et al (6) concluded that peanut oil is highly resistant to autoxidation because of its high percentage of oleic acid and tocopherol content.

All of the fungi studied produced large amounts of free fatty acids. This showed that these fungi had high lipase activity. Since the peanut substrate contained about 50% oil, high lipase activity continued through the entire 8 weeks of fungus growth. Formation of large amounts of free fatty acids was not associated with a comparable decline in total oil except for *A. tamarii*. This is evidence that *A. tamarii* was utilizing the fatty acids as a carbon source. The small decrease in organic matter at the 4- and 8-week intervals that resulted from growth of the *A. glaucus* group and *P. citrinum* indicated that these fungi utilized fatty acids very slowly. These results agree with studies of Goodman and Christensen (8) that show that species of *Aspergillus* and *Penicillium* differ in their utilization of fatty acids as a carbon source.

Fungi used in this study had been previously established (5) as the predominant species in stored peanuts and were the same as those found on other seeds, or closely related. The biochemical changes produced by these fungi on autoclaved peanuts were of the type associated with seed deterioration. The high free-fatty-acid production by these fungi was similar to the increases in fatty acids found by Stansbury (22) and Ward (23) in peanut storage studies at varying moistures. Other deteriorative changes were loss in or-

ganic matter, decreases in total oil, loss of sucrose, and increase in unsaturated fatty acids. Thus, the data indicate that storage fungi are involved in the deterioration of stored peanuts.

LITERATURE CITED

1. Altschul, A. M. 1948. Biological processes of the cottonseed. *In* Cottonseed and cottonseed products. A. E. Bailey, ed. Interscience, New York. p. 157-212.
2. American Oil Chemists' Society. 1952. Official and tentative methods of the American Oil Chemists' Society. V. C. Mehlenbacher, ed. of analytical methods, Chicago.
3. Christensen, C. M. 1957. Deterioration in stored grains by fungi. Botan. Rev. 23:108-134.
4. Cochrane, V. W. 1958. Physiology of fungi. John Wiley and Sons, New York.
5. Diener, U. L. 1960. The mycoflora of peanuts in storage. Phytopathology 50:220-223.
6. Fore, Sara P., Nelle J. Morris, C. H. Mack, A. F. Freeman, and W. B. Bickford. 1953. Factors affecting the stability of crude oils of 16 varieties of peanuts. J. Am. Oil Chemists' Soc. 30:298-301.
7. Foster, J. W. 1949. Chemical activities of fungi. Academic Press, New York.
8. Goodman, J. J., and C. M. Christensen. 1952. Grain storage studies. XI. Lipolytic activity of fungi isolated from stored corn. Cereal Chem. 29:299-308.
9. Henick, A. S., M. F. Benca, and J. H. Mitchell, Jr. 1954. Estimating carbonyl compounds in rancid fats and foods. J. Am. Oil Chemists' Soc. 31:88-91.
10. Holman, R. T. 1954. Autoxidation of fats and related substances. *In* Progress in the chemistry of fats and other lipids. Vol. 2. Academic Press, New York.
11. Hove, E. L., and Z. Hove. 1944. The chemical estimation of "a" tocopherol and total tocopherol in mixtures of the "a," "b" and "g" forms. J. Biol. Chem. 156:601-610.
12. Joslyn, M. A. 1950. Methods in food analysis applied to plant products. Academic Press, New York. p. 405-440.
13. Kirk, P. L. 1936. Kjeldahl method for nitrogen. Anal. Chem. 8:223-225.
14. Lange, W. 1950. Cholesterol, phytosterol, and tocopherol content of food products and animal tissues. J. Am. Oil Chemists' Soc. 27:414-422.
15. Milner, M. 1950. Biological processes in stored soybeans. *In* Soybean and soybean products. Vol. I. K. S. Markley, ed. Interscience, New York. p. 483-501.
16. Milner, M., and W. F. Geddes. 1946. Grain storage studies. III. The relation between moisture content, mold growth, and respiration of soybeans. Cereal Chem. 23:225-247.
17. Milner, M., and W. F. Geddes. 1954. Respiration and heating. *In* Storage of cereal grains and their products. J. A. Anderson and A. W. Alcock, eds. Am. Assoc. of Cereal Chemists. Monograph Series, Vol. II. St. Paul, Minnesota. p. 152-220.
18. Moore, R. N., and W. G. Bickford. 1952. A comparative evaluation of several antioxidants in edible fats. J. Am. Oil Chemists' Soc. 29:1-4.
19. Morris, S. G. 1954. Fat rancidity, recent studies on mechanisms of fat oxidation in its relation to rancidity. J. Agr. Food Chem. 2:126-132.
20. Nagel, C. M., and G. Semeniuk. 1947. Some mold-induced changes in shelled corn. Plant Physiol. 22:20-33.
21. Semeniuk, G. 1954. Microflora. *In* Storage of cereal grains and their products. Am. Assoc. of Cereal Chemists. Monograph Series, Vol. II. J. A. Anderson and A. W. Alcock, eds. St. Paul, Minnesota. p. 77-151.
22. Stansbury, M. F. 1947. Storage of cottonseed and peanuts under conditions which minimize changes in chemical composition. J. Agr. Research 75:49-61.
23. Ward, H. S., Jr. 1955. The effect of moisture and temperature during storage on the germination, respiration, and free fatty acids of Dixie Runner peanuts. J. Alabama Acad. Sci. 27:96-97.

TRANSMISSION OF GEOTRICHUM ROT OF TOMATO FRUIT BY DROSOPHILA MELANOGASTER

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SUMMARY

Geotrichum rot, an important rot of canning tomatoes in the central valley of California, is transmitted by *Drosophila melanogaster*. The pathogen *Geotrichum candidum* was isolated from flies collected in tomato fields and other agricultural areas. Healthy wounded fruit in 15-L jars became rotted when field collections of vinegar flies were introduced but remained healthy without flies. Likewise, healthy wounded fruit in cheesecloth bags in the field remained healthy while unbagged fruit developed Geotrichum rot. As the proportion of flies carrying *G. candidum* increased in the field, the amount of Geotrichum rot also increased. *G. candidum* was also isolated from *Diabrotica duodecipunctata* Fabr., *Apis mellifica* and *Carpophilus hemipterus*, but these insects were never observed to visit sound tomato fruit.

Introduction.—It is estimated that thousands of tons of ripe tomatoes are lost annually to the California canning tomato industry from rots caused by a complex of fungi (1). *Geotrichum candidum* Lk. ex Pers. emend Carmichael is an important and conspicuous pathogen in this complex. It is frequently the sole cause of rot, though often in association with other fungi (2).

Table 1.—Isolation of *Geotrichum candidum* from vinegar flies trapped at different times in Yolo and Sutter counties, California.

Source of flies	County and date	Isolations per flies sampled
peach orchard	Yolo August, 1957	2/8
" "	" "	6/107
" "	" "	19/122
" "	" "	2/186
" "	Sutter Sept., 1957	22/42
" "	" "	38/98
grape vineyard	Yolo August, 1957	1/34
dairy barn	" "	2/130
tomato field	" Sept., 1959	30/30
" "	" "	15/19

The fungus enters the fruit through wounds and growth cracks. Distribution in tomato fields is general. In the central valley of California, the fungus is most prevalent from about September 15 until the completion of harvest, in late October or early November.

That the vinegar fly, *Drosophila melanogaster* Meig., is responsible for transmission of Geotrichum rot was suggested by the scattered pattern of rot distribution and the large numbers of vinegar flies in tomato fields when rotted fruit are most numerous. Herein are the results of observations and experiments testing this hypothesis.

Materials and methods.—Two methods were used in trapping flies. In the first, slices of ripe tomato fruit were placed in the bottom of sterile 500-ml jars. A dilute suspension of yeast cells (brewers' yeast) was poured over the sliced fruit, and the sealed jars were then incubated 12–15 hours at 25°C. The jars were then opened and left at selected locations from about 9 A.M. to noon. Flies, attracted to the tomato-yeast bait, were harvested by inverting a sterile jar over the trap jar. A second method, useful when fly populations were high, was to spread a piece of cheesecloth about one meter square over the top of tomato plants or other material. The center of the cloth was raised at both ends so as to form an inverted V. The vines were then shaken. When enough flies rose into the cloth, it was folded quickly and tightly so as to limit their movement. The flies in both types of trap were moved to the laboratory within 1–5 hours, killed by storage at –15°C for one hour, and plated on NPDA (potato-dextrose agar containing 100 μg/ml of novobiocin) (3). Flies were inactivated for transfer among experimental containers by chilling for about 30 minutes at 3–5°C. All the tomato fruits were of the cultivar Pearson.

In this paper "transmission of rot" is used to mean that the vector carries the pathogen and inoculates it into a susceptible host, with infection and disease resulting. Thus the disease is perpetuated through the medium of the vector. The writer is aware of other meanings for transmission, but the foregoing definition is practical, and precedent for its use is ample (7).

Results.—*Association of G. candidum with vinegar flies.*—One of the first steps in determining the importance of the vinegar fly in rot transmission was to associate the fungus with the fly in agricultural areas. This was done by trapping flies in areas suspected of harboring populations of *G. candidum*. Flies were trapped in 2 counties and plated on NPDA. The cheesecloth-tent trapping method was used in tomato fields and peach orchards of Sutter County and the yeast-tomato bait trap at other locations. The results are summarized in Table 1. A portion of the flies at each trapping yielded *G. candidum*, and it is clear that vinegar flies in substantial numbers do carry *G. candidum*.

Transmission of rot.—Evidence that *D. melanogaster* transmits Geotrichum rot was obtained in the laboratory and in the field. Flies for laboratory tests were obtained from 3 sources: from the University of California campus at Davis, a tomato field at Courtland, California, and a tomato field at Davis. The first two collections were made in August, 1957, and the last on October 4, 1957. Platings of flies made on October 3 and 15 in the Davis field indicated that over 90% of the flies carried *G. candidum* (Table 2).

Fruit to be used for transmission tests were dipped 2–3 minutes in 1% sodium hypochlorite, and cut with a blade to produce a wound 1–2 cm long and extending through the pericarp wall into the locule.

In the tests in August, 10 fruit were placed in each of three 15-L jars. Ten flies from the Courtland collection were introduced into 1 jar, and 10 flies from the Davis collection in another; the third jar contained only wounded fruit. Methods were similar in October except that 10 fruit were also inoculated with *G. candidum*.

In both tests fruit in jars containing the vinegar flies (Fig. 1-A) and fruit inoculated with *G. candidum*

Table 2.—Proportion of *Drosophila melanogaster* carrying *G. candidum* and prevalence of Geotrichum rot in 2 tomato fields.

Date	Per cent flies with *G. candidum*[a]		Geotrichum index[b]	
	1957	1958	1957	1958
Aug. 26		50(40)		
Sept. 3		67(116)		5
10		59(44)		
17	33(51)		25	
19	33(112)		25	
20		76(229)		41
21		82(101)		
25			157	
28		89(168)		
Oct. 1		81(59)		62
2		85(78)		
3	95(124)		376	
10		97(208)		260
15	98(95)		794	

[a] Figures in parentheses are the number of flies tested in establishing the percentage shown.

[b] The number of fruit rotted by *G. candidum* (in five 80-ft rows in 1957 and five 40-ft rows in 1958).

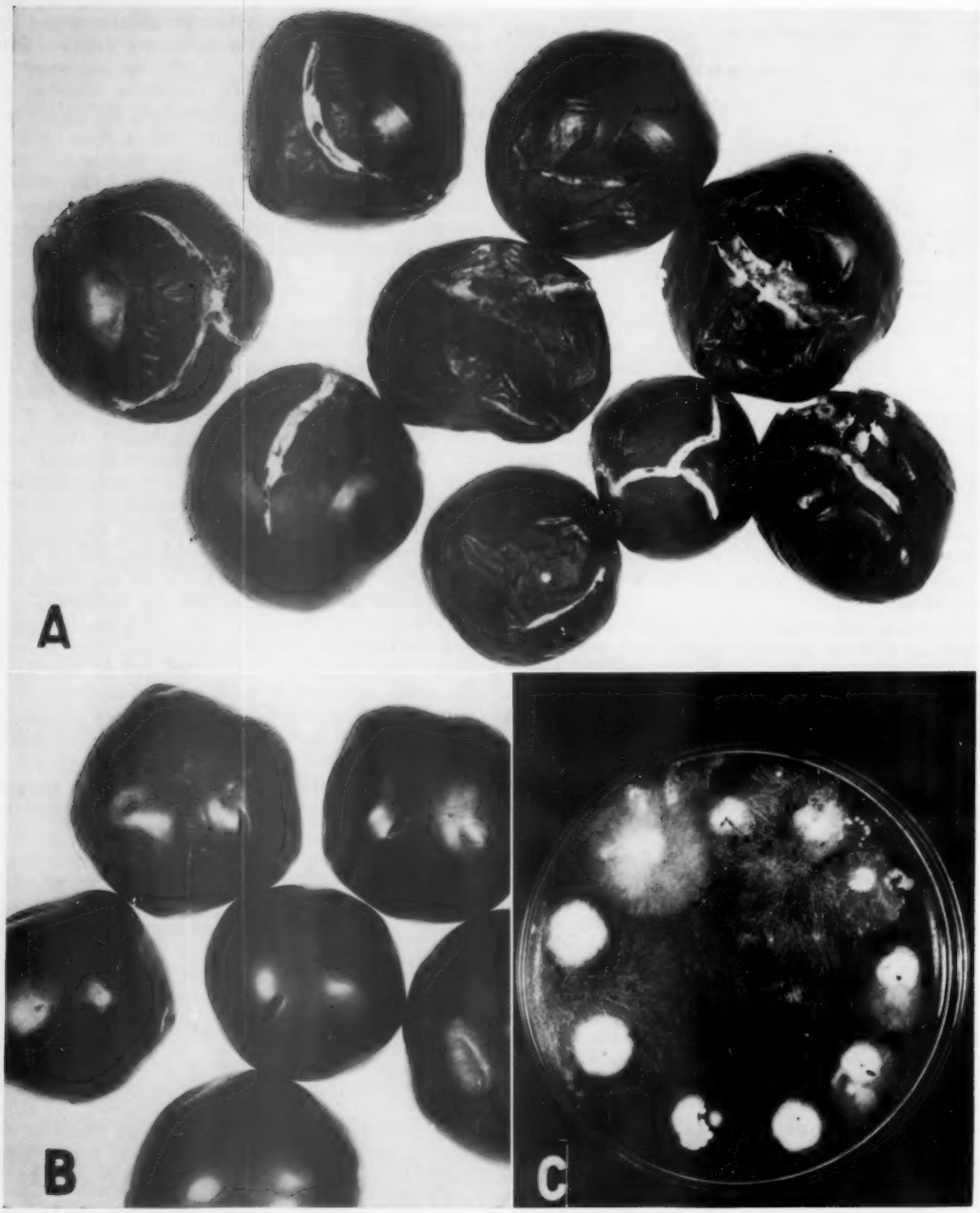

Fig. 1.—A) Tomato fruit wounded and incubated in 15-L glass jar with vinegar flies from a tomato field. Note white mycelial growth of *Geotrichum candidum* and adjacent watery tissue. **B)** Uninoculated control, ×½. **C)** Vinegar flies collected at Davis in October, 1958, and plated on NPDA. Circular dense white colonies are *G. candidum*.

were severely rotted in 5 days, whereas all control fruit were healthy (Fig. 1-B).

The flies made frequent visits to the freshly made wounds, starting within 5 minutes of introduction into the jars. Two to 3 days after these visits the wounds enlarged in length and width (Fig. 1-A), and the white mycelium of *G. candidum* was macroscopically visible.

The laboratory trials and data previously given showed that not only does *D. melanogaster* harbor *G. candidum* but that it is an effective agent of inoculation. Experiments were made next to determine if the flies transmit the disease in the field.

On October 18th, 1957, when nearly all the vinegar flies in a field at Davis carried *G. candidum* (Table 2), 25 ripe fruit on the vine in widely separated areas of a field were covered with a cheesecloth bag and sealed around the stem so as to exclude insects. Before bagging, the fruit wall was swabbed with cotton saturated with 70% alcohol and a cut was then made, 1 mm deep and 10 mm long, in the fruit wall. Such a wound simulates a shallow natural growth crack. Many natural growth cracks extend into the locule. As a control, 25 fruit were wounded similarly and left unbagged. Fruit selected for this and similar tests were on the north side of vines and not directly under a canopy of leaves. Wounds were made and fruit bagged between 1 and 3 P.M. Within 10 minutes, flies were seen to visit the wounded fruit. At 48 hours after wounding, all of the wounds seemed dry, with no sign of developing rot, but at 72 hours 23 of the 25 unbagged fruit were rotting at the site of the wound. Five days after the test was started the bagged fruits were still healthy, whereas 23 of the 25 outside the cheesecloth were rotted. The white mycelium typical of *G. candidum* was present on the growth cracks of several of the fruit.

The experiment was repeated on September 17, 1958, with results essentially the same. Watery tissues typical of Geotrichum rot around the wound were first observed in unbagged fruit 72 hours after wounding. Of the bagged fruit, 23 were healthy, and 2 rotted. Twenty-one outside the bags were rotted, and 4 were healthy. After 5 days all rotted fruit were placed in sterile sealed jars in the field and removed to the laboratory, where diseased tissue was plated on NPDA. *G. candidum* was obtained from 23 fruits. In the case of 10 fruits it was the only pathogen isolated.

A third bagging experiment was initiated on September 24, 1958. Flannel bags with pores small enough to exclude the smallest insects replaced cheesecloth bags, and the fruit were wounded into the locule. Twenty-four fruits were bagged and 24 were unbagged. The results at 5 days were as follows: 23 bagged fruit were healthy, and one was infected by a species of *Rhizopus;* unbagged fruit were all rotted, and *G. candidum* was identified from each.

Habits of D. melanogaster and disease transmission.—Several investigators have pointed out the importance of yeast and other microorganisms as a food for *Drosophila* spp. (4, 5, 13). In nature the flies feed on yeasts, and in California tomato fields large numbers of flies are found on ripe fruit, which abound with yeasts and various other fungi. Although *D. melanogaster* feeds on decaying fruit, it seems to oviposit in the moist healthy tissue of ripe fruit. Michelbacher and Middlekauff (8) observed as many as 172 eggs deposited in a single freshly wounded fruit in one hour. The ovipositor, mouth parts, and legs contact the wound tissue. Considering that flies also visit decaying fruit, it seemed likely that *G. candidum* would be deposited in healthy tissue at least by flies that had previously visited fruit containing *G. candidum*.

Further evidence that flies carry the fungus and can deposit it by walking over a surface was obtained in the following manner: the heads and legs of field flies were removed, with sterile techniques, under the dissecting microscope and plated on NPDA. *G. candidum* was isolated from 10 of 36 heads and 2 of 27 legs. Further, flies collected in a peach orchard at Davis on August 9, 1957, were allowed to walk on the surface of agar in Petri dishes for 5 minutes. Seven colonies of *G. candidum* were obtained from 133 flies distributed in 9 plates. These findings prove conclusively that *D. melanogaster* harbors the pathogen and can deposit it by contacting a moist surface.

Air dissemination of spores.—To test the possibility that spores of *G. candidum* are carried by air currents, and thus could fall in susceptible wounds directly from the air, plates of potato-dextrose agar (PDA) and V-8-juice agar (9) were exposed in a tomato field at Davis. Dates of exposure were September 24 and 26 and October 1, 3, 8, 10, 15, and 17, 1958, at 9 A.M., 12 noon, and 4 P.M. on each date. Plates were opened for one hour and a total of 390 plates were employed. Table 2 shows data on the per cent of flies carrying *G. candidum* and the incidence of rot in the same field.

The pathogen was present in the air, but apparently in very low numbers. Only 10 colonies were obtained—one on each of 5 different dates and 5 on October 10. Neither medium, time of day, nor date influenced the number of colonies collected in the plates. No colonies were obtained from media exposed on October 15 and 17, when Geotrichum rot was most prevalent in the field. If the plate technique is a reliable method for sampling the numbers of spores in the air, then the amount of *G. candidum* was too low to account for the amount of rot in the field or the increase in rot during the period of air sampling. If spores fell uniformly over a 24-hour period, then only .0004 spore fell per sq cm. These findings and the fact that wounded fruit within cheesecloth bags remained healthy, though exposed in the field for 5 days, strongly suggest that air-borne spores are not an important source of inoculum for Geotrichum rot.

Proportion of flies carrying G. candidum and the amount of rot.—Since previous results had shown that the vinegar fly plays a major role in the inoculation of

susceptible tomato fruits with *G. candidum*, it seemed important to determine the relationship between the proportion of flies carrying *G. candidum* and the amount of rot in tomato fields.

For this purpose two plots were established at Davis—one in 1957, the other in 1958. Each plot measured about 300 x 75 ft. Twelve traps were placed in each field—4 equidistant the length of each outside and center row, on each of several dates (Table 2). The fruits rotted by *G. candidum* were counted in 5 rows, each 80 ft long, in 1957, and 5 rows, each 40 ft long, in 1958. Fruit were not harvested in either field. In 1957, weather conditions were favorable for rot development. Rain occurred on September 27, 28, 29, and 30, and on 9 of the first 15 days in October. In 1958, however, the growing season was hot and dry; only .06 in. rain fell in the period Sept. 1 to Oct. 15. Thus, to simulate rain, which increases fruit cracking, the 1958 plot was sprinkler-irrigated for 0.5, 2, and 3 hr on Oct. 7, 8, and 9, respectively.

The results (Table 2) show that both the proportion of flies carrying *G. candidum* and the number of fruit with Geotrichum rot increased as the season advanced.

Discussion.—Although the vinegar fly has long been recognized as a pest in processing plants and in the field (6, 8, 10), mainly because it oviposits in tomato fruit, little has been known of the potential importance of the fly in the epiphytology of tomato fruit rots.

This paper shows the effectiveness of the vinegar fly in transmitting a single fruit rot, but it is probable that other pathogens may be associated with the vinegar fly in much the same way. *Rhizopus* spp. and *Mucor hiemalis* have been isolated from the vinegar fly in the course of the present work.

Before the importance of vinegar flies in the total rot picture can be adequately assessed, it will be necessary to determine which rots are transmitted by the flies, their epiphytology, and effective means of controlling the flies in tomato fields.

Geotrichum candidum seems to be more intimately associated with *Drosophila* spp. than are other pathogens causing tomato fruit rot. The nature of its mycelium is probably an important key to this association. The hyphae fragment into arthrospores so that the cheesy white growth observed in fruit tissues consists of individual cells and short hyphal fragments. Thus, great numbers of single cells embedded in or on a soft matrix are available for distribution by the fly. On the other hand *Rhizopus stolonifer* and *Mucor hiemalis* frequently rot fruit without producing any reproductive elements available for dissemination by the vinegar fly. In addition there is some evidence that *Drosophila* spp. feed on hyphae of *G. candidum* or at least ingest it, for it was isolated from the digestive tract of wild flies (11, 12, 14).

The tendency of the adult vinegar fly to visit and oviposit in healthy fruit and feed on the microorganisms in decaying tissues ensures the spread of Geotrichum rot in tomato fields. Transmission is also assured by egg deposition in healthy tissue. Eggs develop into larvae in 1.5–3.0 days. As the larvae develop they feed on yeasts and other microorganisms deposited by the adult fly. I have observed numerous fruit with seething masses of larvae in natural growth cracks.

As the growing season advances, the fly population becomes more efficient in transmitting Geotrichum rot–through greater numbers of flies, a greater proportion carrying *G. candidum*, and greater numbers of infection sites.

Though the vinegar fly seems to be responsible for inoculating tomato fruit with *G. candidum*, entrance of the pathogen doubtless occurs to some extent through natural growth cracks in direct contact with the soil, air-borne inoculum, and the splashing of rain and dew onto growth cracks. I have isolated the pathogen from several agricultural soils and tomato foliage, stems, calyxes, and blossoms (unpublished data). Studies on the epiphytology of Geotrichum rot are still in progress.

Vinegar flies probably disseminate *G. candidum* over relatively large areas. Data already presented show that flies from several sources carry the pathogen. Michelbacher and Middlekauff (8) suggested that flies migrate from peach orchards to tomato fields after the peach harvest. By tagging flies with radioactive phosphorus Warner (15) showed that flies migrate at least 2 miles. It seems highly probable that populations of *D. melanogaster* migrating to tomato fields in August carry with them the inoculum necessary for the initiation of Geotrichum rot.

The question may be raised regarding the transmission of Geotrichum rot by other insects. The pathogen was isolated from the twelve-spotted cucumber beetle, *Diabrotica duodecipunctata* Fabr., the honey bee, *Apis mellifica* L., the dried fruit beetle, *Carpophilus hemipterus* L., and a species of Leptocera, but during many hours of observation in tomato fields none of these insects were seen to visit growth cracks or other sites suitable for inoculation of the pathogen into healthy fruits.—Department of Plant Pathology, University of California, Davis.

LITERATURE CITED

1. Butler, E. E. 1959. Fungi and rots in California canning tomatoes. Plant Disease Reptr. 43:187-192.
2. Butler, E. E. 1960. Pathogenicity and taxonomy of Geotrichum candidum. Phytopathology 50:665-672.
3. Butler, E. E., and R. B. Hine. 1958. Use of novobiocin for isolation of fungi. Soil Science 85:250-254.
4. de Camargo, R., and H. J. Phaff. 1957. Yeasts occurring in Drosophila flies and in fermenting tomato fruits in northern California. Food Research 22: 367-372.
5. Delcourt, A., and E. Guyenot. 1910. De la possibilité de'etudier certain Depteres en milieu definite. Compt. rend. 151:255-257.
6. Ditman, L. P., E. N. Cory, and A. R. Buddington. 1936. The vinegar gnats or pomace flies—their relation to the canning of tomatoes. Maryland Univ. Agr. Expt. Sta. Bull. 400:91-111.
7. Leach, J. G. 1940. Insect transmission of plant diseases. McGraw-Hill Company, New York. 615 p.

8. Michelbacher, A. E., and W. W. Middlekauff. 1954. Vinegar fly investigations in northern California. Econ. Entomol. 47(5):917-922.
9. Miller, P. M. 1955. V-8 juice agar as a general-purpose medium for fungi and bacteria. Phytopathology 45:461-462.
10. Pepper, B. B., J. P. Reed, and O. Starnes. 1953. Drosophila as a pest of processing tomatoes. New Jersey Agr. Expt. Sta. Bull. 266:1-8.
11. Phaff, H. J., M. W. Miller, and M. Shifrine. 1956. The taxonomy of yeasts isolated from Drosophila in the Yosemite region of California. Antonie van Leeuwenhoek J. Microbiol. Serol. 22:145-161.
12. Phaff, H. J., M. W. Miller, J. A. Recca, M. Shifrine, and E. M. Mrak. 1956. Studies on the ecology of Drosophila in the Yosemite region of California. II. Yeasts found in the alimentary canal of Drosophila. Ecology 37:533-544.
13. Shehata, A. M. El-Tabey Awab, and E. M. Mrak. 1951. The fate of yeast in the digestive tract of Drosophila. Am. Naturalist 85:381-383.
14. Shehata, A. M. El-Tabey, E. M. Mrak, and H. J. Phaff. 1955. Yeasts isolated from Drosophila and from their suspected feeding places in southern and central California. Mycologia 47:799-811.
15. Warner, R. M. 1959. Radioactive tagging for tracing movements of Drosophila. Proc. 13th Ann. Research Conf. Calif. Fig. Inst. :35-37.

METHOD OF ISOLATION OF ERWINIA TRACHEIPHILA AND AN IMPROVED INOCULATION TECHNIQUE

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SUMMARY

In greenhouse tests, *Erwinia tracheiphila* was successfully isolated from cucurbits by 2 methods. In method A, a small quantity of sterilized water was injected into the petiole of a wilted leaf by means of a hypodermic needle and slowly withdrawn and discharged into a Petri dish containing nutrient agar. In method B, a loop-type inoculating needle was glided along the surface of a longitudinally cut petiole of an infected leaf and streaked on nutrient agar.

The multi-needle puncture inoculation method gave consistently satisfactory results under greenhouse and field conditions. Headless pins (25–30) are inserted into a cloth and cotton-padded rubber stopper, which is dipped in a suspension of the pathogen and forced through the cotyledon of the cucumber seedling. Under 2 temperature and humidity levels the multi-needle puncture method was more effective than the leaf-rub inoculation method.

Literature review.—Ervin F. Smith (6, 7) in his original work on bacterial wilt of cucurbits indicated that it was isolated in pure culture from the interior of cucumber stems. He pointed out the difficulty of isolating the pathogen by the poured-plate practice, attributing this to the difficulty of obtaining a suspension of the slime in which the bacteria were embedded. He suggested cutting the stem of an infected plant at right angles and keeping the severed pieces in a straight line slowly pulling them apart. The sticky exudate forms cobweb-like threads containing the bacteria. A sterilized needle is then touched to the strands and subsequently placed in a tube of broth for incubation. He further indicated that frequent transfers on culture media were necessary and that the organism could be kept alive longest in milk or in sugar peptone water over calcium carbonate. Burkholder (1) isolated the bacterium by inserting a sterile needle into the vascular tissue of the cut end of a stem of an infected plant and removing and streaking on nutrient agar.

Various techniques have been used for introducing the pathogen into cucurbit plants. Smith (6) introduced the culture by making 20–30 needle pricks in the center of the lamina. In a series of over 700 such inoculations he obtained a percentage of infection on susceptible plants ranging from 75 to 100%. Harris (3) inoculated cucumber and muskmelon plants with *E. tracheiphila* by making needle punctures in the stem. Williams and Lockwood (10) found that the leaf-rub and hypodermic needle injection methods were both effective means of inducing bacterial wilt. They reported further that disease incidence from the former method was 80–100% and did not differ significantly from incidence from the latter. Wilson et al (11) inoculated cucumber plants with a No. 20 hypodermic needle at 2 points, about 1½ and 2½ in. from the ground. The inoculum was forced into the upper hole until it exuded from the lower. Nuttal and Jasmin (4) in studying the inheritance of bacterial wilt resistance in cucumber inoculated at the first-true-leaf stage with a B-D Yale syringe using a 1-in. hypodermic needle. The needle was inserted under the upper epidermis of a cotyledon and a few drops of bacterial suspension introduced into the seedlings. Wei et al (9) introduced the inoculum into the hypocotyl of cucumber plants with a small cotton swab through punctures made by a group of 5 fine entomological pins.

The effect of environment on inoculation has been studied by Wei et al (9) who showed that day-length was not an influencing factor on the rate of development of bacterial wilt in cucumber, and found no significant differences between 12 and 18 hours of daylight.

Rand and Enlows (5) found very little difference in percentage of infection when greenhouse inoculations were made under wet or dry conditions or between those made in sunlight or shade. Nuttal and Jasmin (4) from greenhouse inoculation tests reported: "During periods of bright sunny weather symptoms of bacterial wilt appeared 10–12 days following inoculations, while the development of the disease was delayed by as much as an additional 2 weeks when the weather was consistently dull and cool."

Because of certain shortcomings of those methods, investigations were begun to find better ways of isolating the pathogen and inoculating plants with it.

Materials and methods. — *Isolation.* — Cucumber seedlings of a known wilt-susceptible variety, WSMR 18, were inoculated in the cotyledons with a culture suspension of *E. tracheiphila*, obtained from Dr. W. H. Burkholder, and the disease was allowed to develop. Initial symptoms of infection normally develop on the inoculated cotyledon 4–6 days after inoculation; it usually required 4 additional days for secondary infection to appear (always on the first true leaf). For isolation or recovery of the pathogen, infected plants showing pronounced wilting of at least 1 true leaf were selected and 2 different methods were employed.

In method A, the plant was flamed over a Bunsen burner and maintained aseptically. A sterilized hypodermic syringe of 1 ml capacity with a No. 20 needle was filled with 0.5 ml of sterilized water. The needle was inserted near the node into the vascular tissue of the petiole in the direction of the wilted leaf, and one-half of the water content was injected and then withdrawn by suction. The material thus obtained was placed on nutrient agar in a Petri dish and incubated for 2–3 days at 80°F (Fig. 1).

In method B the wilted plant was flamed as above and the petiole of the wilted leaf was aseptically detached from the stem and cut longitudinally with a sterilized knife. A loop-type inoculating needle, flamed and dipped in a drop of sterile water, was then glided along the cut surface and streaked on a nutrient agar plate. The incubating period and temperatures were the same as with method A.

Inoculation.—1) *Multi-needle puncture.*—Eight-to ten-day-old cucumber seedlings grown in flats in sterilized soil were normally used in the greenhouse experiments.

An assembly, made by inserting the pointed ends of 25–30 headless pins into a cloth and cotton-padded rubber stopper, was dipped in a suspension of the pathogen and pricked through the cotyledon supported from below with a 4-in. pot label (Fig. 1).

2) *Leaf-rub.*—Cotyledons of cucumber plants 8–10 days old were dusted with 300-mesh Carborundum.

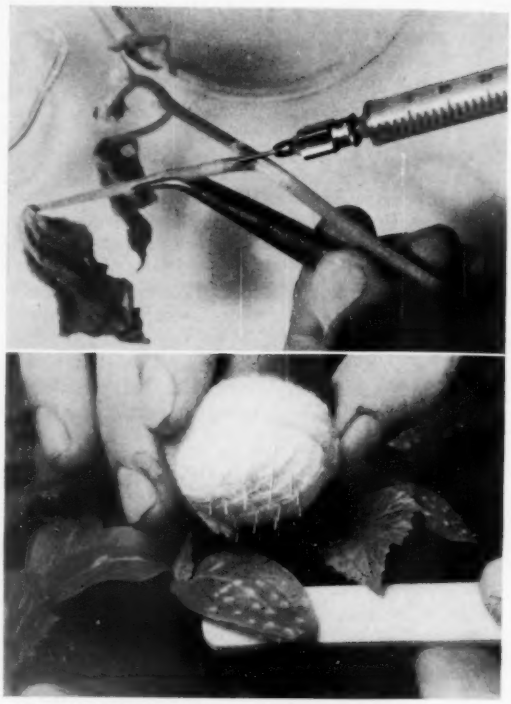

Fig. 1.—Above: Isolation of *Erwinia tracheiphila* from the petiole of a wilt-infected cucumber plant with hypodermic syringe and needle. **Below:** Inoculation of young cucumber plant with *Erwinia tracheiphila* by the multi-needle puncture method.

The suspended culture was introduced into the plant by rubbing a culture-soaked cotton dauber on the dusted cotyledon.

The inoculum was a 3-day-old culture of *E. tracheiphila* suspended in distilled water and adjusted to a turbidity of about 80% transparency on a Cenca photelometer using a 525-μ filter. The inoculating medium was stirred frequently to prevent the colloidal suspension from settling.

The efficiency of the two inoculation techniques was compared in a series of tests under different temperature and humidity levels.

In experiment A, one group of cucumber seedlings inoculated by both methods was allowed to incubate under a fairly constant 65° F and another was kept night and day, respectively, at 60° and 80°F.

In experiment B, plants were inoculated by the same 2 methods. One group was alowed to incubate at 35–65% relative humidity, and the other was placed for a 60-hour period in an incubation chamber at a relative humidity of 90–100%.

To determine the effect of day length on rate of wilt development, plants were inoculated in 2 separate tests by the multi-needle puncture method. One-half of the inoculated plants were permitted 8 hours

of natural daylight, the others were given supplemental artificial light for 16 hours daily (a 400-watt mercury arc vapor lamp, G. E. No. H400-R1 LS-103, about 3 feet above the plants).

Symptoms on infected plants were recorded periodically beginning when about half the plants showed initial symptoms of primary infection.

Results and discussion.—*Methods of isolation.*—In subsequent isolations from 18 wilted plants, made by method A, about 66% yielded the pathogen in pure form while of the remaining 34% only 6% were severely contaminated. The 47 isolations made during the same period by method B yielded 85% pure cultures, with about 15% being only slightly contaminated. Thus, both methods were very effective. Virulence and identity of the organism were determined by reinoculating the susceptible host with the pathogen. In all instances the wilt symptoms typical of that organism were reproduced and the organism itself recovered. Although all isolates in a series of tests were pathogenic, there were differences between some isolates of individual plants in time from inoculation to development of symptoms.

The syringe method is a modification of a technique (8) used for recovery of peritoneal fluid in animals. In the plant or leaf petiole the quality of material in liquid form is small, or the bacteria may often be embedded in a viscid material; therefore, flushing by injecting distilled water is desirable. The water held back in the syringe is discharged along with the withdrawn material to suspend the latter and also to flush the needle. When plants are 15–20 days old, stringiness and insolubility of the diseased tissue are not a problem. The syringe method might also be well worthy of trial on fruit or other succulent plants where the organism is deep-seated and the disease appears to be systemic.

Methods of inoculation.—Of various methods of introducing the pathogen, (3, 4, 6, 9, 10, 11) into the cucumber plant, 2 have been used by the authors between 1954 and 1958 with varying success. The hypodermic needle stem injection method of Wilson et al (11) used here in 1954–56 was found impractical for inoculating young seedlings, because of mechanical damages to the stem. It was replaced in 1957 by the leaf-rub method (10). Susceptible lines thus inoculated have shown a wide variation in percentage of infection in different experiments as well as in replications within an experiment. In the summer of 1958 several replications of a known wilt-susceptible variety with a total of 408 plants, after repeated inoculations, produced 25–100% infection. In the greenhouse this method proved difficult and results have been inconsistent. In the spring of 1959, with development of the multi-needle method, 660 plants of the same variety became 100% infected in a relatively short time. Repeated inoculation in the greenhouse on many breeding lines has given consistently satisfactory results. For better evaluation of the two methods, comparative tests under 2 temperaturees and 2 humidity levels were conducted, in the greenhouse, in the fall and spring of 1959 and 1960.

Table 1.—Percentage of infected plants 12 and 18 days after inoculation, inoculated with *Erwinia tracheiphila* by two different methods and incubated at two temperature levels.

Inoculation method and medium	Incubation at 60–80°F			Incubation at 65°F		
	No. plants inoc.	% infected plants/days after inoc.		No. plants inoc.	% infected plants/days after inoc.	
		12	18		12	18
Multi-needle, culture	84	67	99	85	12	73
Multi-needle, water	55	0	0	56	0	0
Leaf-rub, culture	85	1	6	84	0	1
Leaf-rub, water	54	0	0	56	0	0

Table 1 compares the percentage of infected plants with the 2 methods at 2 incubating temperatures. The relatively low virulence of the culture in this experiment caused a considerable delay in the development of the initial symptoms, but this effect was most obvious when plants were inoculated by the leaf-rub method. At 60° and 80°F, respectively night and day, 99% of the plants were infected by the multi-needle method 18 days after inoculation, compared with 6% for the leaf-rub method. Disease development at a constant 65°F was considerably slower, with readings of 73% for the former and 1% for the latter. Seedlings inoculated with water blank controls showed no apparent damage.

The low percentage of infection introduced in this experiment by the leaf-rub method raised the possibility that the dry air in the greenhouse during incubation might have affected one group of plants more than the other. Coons (2) thought that hot, dry summers might do much to control the spread of the dis-

Table 2.—Rate of development of wilt symptoms on cucumbers inoculated with *Erwinia tracheiphila* by two different methods and kept under two humidity levels. Inoculation date March 28, 1960.

Inoculation method	Treatment relative humidity	Percentage[a] of primary and secondary infection at various dates					
		4/1	4/4	4/8		4/12	
		P[b]	P	P	S[c]	P	S
Multi-needle	35–65%	25	90	100	68	100	96
Leaf-rub	35–65%	0	0	7	0	36	14
Multi-needle	90–100% (60 hours)	64	100	100	71	100	100
Leaf-rub	90–100% (60 hours)	0	0	21	4	46	32

[a] Based on 28 plants per method per treatment.
[b] P = primary infection—normally on the inoculated cotyledon.
[c] S = secondary infection—on the first true leaf.

Table 3.—Rate of disease development on cucumber plants inoculated with *Erwinia tracheiphila* and kept under two different light conditions.

Treatments	Test series	No. plants inoc.	% plants with primary infection—days after inoc.			% with secondary infection
			6	8	13	13 days
Natural daylight (8 hours)	I	109	33	85	100	91
Supplemental light (16 hours)	I	112	46	80	99	90
			4 days	6 days	10 days	
Natural daylight (8 hours)	II	112	26	95	100	100
Supplemental light (16 hours)	II	111	41	96	100	94

ease by drying out the wounds made by the insects before the bacteria incubated. Thus, infection would be expected to progress more rapidly at high humidity levels than under controlled humidity. The percentage of infected plants (Table 2) in the first readings for the respective methods would indicate that the higher humidity levels favored initial infection, but the differences between treatments within the multi-needle method were nonsignificant at the second reading or when 100% primary infection had developed. The differences in rate of infection between methods or between treatments within the leaf-rub method itself are much more pronounced. On the basis of these tests the multi-needle puncture method was more effective in introducing the disease in less time and was also less influenced by environment than the leaf-rub inoculation method.

Some differences of opinion (4, 5, 9) on the effect of daylight on the rate of development of the disease led to this investigation. In the 2 successive tests where plants inoculated with *E. tracheiphila* by the multi-needle puncture method were allowed to incubate at 2 different day lengths, the rate of infection appeared to indicate a similar pattern.

In both tests (Table 3) the incidence of initial infection under supplemental light appears to be slightly higher in the first reading. No significant difference, however, between treatments is found in all successive readings, including those taken 2 days later, and 100% infection occurs in the same time after inoculation in both treatments. The higher percentage of initially infected plants in the first reading could well be attributed to higher incubation temperatures possibly created by the mercury arc lamp, rather than to light itself. These findings appear in accord with those of Wei et al (9) and Rand (5). Under the conditions of these tests, day length appears not to influence the rate of bacterial wilt development.

The multi-needle puncture method gave consistently satisfactory results and was adaptable to a variety of conditions. The numerous punctures also provide an excellent means of identifying inoculated plants.

LITERATURE CITED

1. Burkholder, W. H. 1960. Some observations on Erwinia tracheiphila, the causal agent of the cucurbit wilt. Phytopathology 50:179.
2. Coons, G. H. 1913. Cucumber and muskmelon wilt. Michigan Farmer 140:1, 2.
3. Harris, H. A. 1940. Comparative wilt induction by Erwinia tracheiphila and Phytomonas Stewarti. Phytopathology 30:625-638.
4. Nuttal, V. W., and J. J. Jasmin. 1958. The inheritance of resistance to bacterial wilt, (Erwinia tracheiphila E. F. Smith, Holland) in cucumber. Can. J. Plant Sci. 38:401-404.
5. Rand, F. V., and E. M. A. Enlows. 1920. Bacterial wilt of cucurbits. U. S. Dept. Agr. Bull. 828:1 32.
6. Smith, E. F. 1911. Wilt of cucurbits. *In* Bacteria in relation to plant diseases 2:209-299. Carnegie Institute of Washington.
7. Smith, E. F. 1920. An introduction to bacterial diseases of plants. W. B. Sanders Co., Philadelphia. 688 p.
8. Society of American Bacteriologists. 1957. Manual of microbiological methods. McGraw-Hill Book Co., New York. 315 p.
9. Wei, C. R., J. C. Walker, and R. P. Scheffer. 1952. Plant nutrition in relation to disease development. VII. Cucurbit wilts. Am. J. Botany 39:245-249.
10. Williams, L. E., and J. L. Lockwood. 1956. Control of bacterial wilt of cucumber by antibiotic sprays. Plant Disease Reptr. 40:479-482.
11. Wilson, J. D., C. A. John, H. E. Wohler, and M. M. Hoover. 1956. Two foreign cucumbers resistant to bacterial wilt and powdery mildew. Plant Disease Reptr. 40:437, 438.

PHYTOPATHOLOGICAL NOTES

OBSERVATIONS ON LYOPHIL PRESERVATION AND STORAGE OF PYTHIUM SPECIES

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Work on the lyophilization of *Pythium* species commenced in April, 1952. The lyophil method for preservation of these organisms was reported as successful in 1954 (1). Of the many types of suspending media examined, only the use of organic plant parts gave satisfactory results with *Pythium* species that produced oogonia. Lyophilization was not successful with those species that produced only mycelium or mycelium and sporangia. The primary method of lyophilization consisted of drying the material over a 3-hour period without prior freezing.

These *Pythium* species had been in storage from 5 years 11 months to 7 years prior to this re-examination. Lyophil tubes, representing each of the oogonial-producing organisms stored, were opened, rehydrated, and the colonized straws plated on the surface of carrot-decoction agar, contained in petri dishes.

In all cases but one, the results were similar to those obtained from the assay conducted in 1953 (Table 1). The last 2 remaining tubes, stored 5 years 11 months and containing straws colonized by *P. debaryanum*, were found non-viable. In 1953, this organism grew from straws contained in similar lyophil tubes. *P. acanthicum* and *P. irregulare*, the 2 species having projections on the oogonial wall, produced viable cultures every time they were examined, 24 and 10 times, respectively. Those spherical oogonial-producing species in storage 6 years 3 months, *P. aphanidermatum* and *P. debaryanum*, and those in storage 6 years 1 month, *P. debaryanum* and *P. splendens*, did not survive. Two isolates of *P. paroecandrum* and 1 of *P. rostratum*, producing spherical oogonia, survived the lyophil processing and the 5 year 11 month storage period. The success with these latter organisms was attributed to the profuse oogonial production prior to lyophilization of these species. Additional time was allowed for production of oogonia in the straws before processing.

The survival of a number of *Pythium* species after lyophil preservation and subsequent storage will enable investigators to handle a number of these organisms as they do those producing smaller spores. Fungi having large specialized spores, as those found in certain *Pythium* species, can be lyophilized and stored for prolonged periods of time.

LITERATURE CITED

1. Staffeldt, E. E., and E. L. Sharp. 1954. Modified lyophil method for preservation of Pythium species. Phytopathology 44:213-215.

Table 1. Assay of *Pythium* species preserved by the lyophil method

Species	Time in storage[a]		Previous assay 1953	Present assay April, 1959
	Years	Months		
P. acanthicum Drechsl.[b]	7	0	+[c]	+
P. acanthicum[d]	7	0	+	+
P. acanthicum[e]	7	0	+	+
P. acanthicum	6	3	+	+
P. aphanidermatum (Edson) Fitzp.	6	3	−	−
P. debaryanum Hesse	6	3	−	−
P. irregulare Buis.	6	3	+	+
P. acanthicum	6	2	+	+
P. acanthicum	6	1	+	+
P. debaryanum	6	1	−	−
P. irregulare	6	1	+	+
P. splendens Broun	6	1	−	−
P. acanthicum	5	11	+	+
P. debaryanum	5	11	+	−
P. irregulare	5	11	+	+
P. paroecandrum Drechsl.	5	11	+	+
P. paroecandrum	5	11	+	+
P. rostratum Butl.	5	11	+	+

[a] Total time in storage to the 1959 assay.
[b] The material was dried for 3 hours at −20°C after being pre-frozen at −45°C.
[c] + = viable cultures were obtained; − = no growth of the organism was obtained.
[d] The material was dried for 3 hours at −10°C after being pre-frozen at −45°C.
[e] The material was dried for 3 hours without prior freezing.

LONGEVITY OF THE BACTERIAL WILT ORGANISM IN ALFALFA HAY, POD DEBRIS, AND SEED

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Jones (5) showed that the bacterial wilt organism (*Corynebacterium insidiosum* (McCull.) H. L. Jens.) could be carried in all vegetative parts of the alfalfa plant. More recently, Cormack and Moffatt (3) demonstrated its occurrence in alfalfa seed. There are few studies, however, on the longevity of the organism in these materials and their possible importance in spreading the disease. Kernkamp and Hemerick (6) isolated virulent cultures from infected tap roots of alfalfa stored in a home freezer at −20°C for 21 months. Cormack, Peake, and Downey (4) found that dried roots and tops of diseased plants stored at room temperature, 5°C, or −20°C for 3 years were as infective as freshly collected material.

Other bacterial pathogens vary in ability to persist in plant materials. For example, Ark (1) showed that *Xanthomonas malvacearum* (E. F. Sm.) Dowson could live for 6 years in dried cotton plants stored at room temperature. Chamberlain (2) preserved *Pseudomonas glycinea* Coerper for 7 years and *P. tabaci* (Wolf & Foster) Stevens for 3½ years in soybean leaves at 5-7°C, but found that neither of these organisms lived beyond 2 months in leaves stored at laboratory temperature.

The present study investigated the longevity and inoculum potential of the bacterial wilt organism in alfalfa materials stored dry at room temperature (70°-80°F). Samples of alfalfa hay from diseased plants in 5 fields near Edmonton, Alberta, were stored in paper bags in the laboratory for 10 years. The stems and leaves in these samples were separated for each test. Also stored were 3 samples of alfalfa pod debris, obtained during threshing of the seed from infected plants in different fields, and 2 samples of the seed. The infective capacity of the organism in all of these materials and samples was determined yearly by using them as inoculum for healthy plants of Grimm alfalfa grown in pots in the greenhouse. Inoculations were made by the root-wound insert method (4) with material pulverized in a mortar and soaked in sterile water for 4 hours. Aseptic precautions were taken to avoid contamination and mixing of materials. Six replicate pots containing 5 plants each were used for each treatment. Control plants, inoculated with similar material from healthy plants, were included in each experiment. Assessment was made by determining the percentage of plants infected and the average disease rating (4), 3 months after inoculation.

Table 1 shows that the bacterial wilt organism in the stem portion of alfalfa hay retained a high degree of viability and infective capacity throughout the 10-year period. Inoculum prepared from the stems was more infective than that from the leaves, initially and at all periods of storage (Tables 1 and 2). In both materials, the infective capacity of the bacteria remained fairly constant for 8 years, and then diminished, particularly in the leaves. After 10 years of storage (Table 2), individual samples differed significantly in infective capacity. Virulent bacteria persisted to the highest degree in the stems of samples 2 and 4. Except in sample 4, infectivity of the leaves was very low after 10 years.

TABLE 1. Development of bacterial wilt in Grimm alfalfa inoculated in the greenhouse with infected alfalfa materials stored dry at room temperature for different periods

Material	Years of storage	% of plants infected	Avg. disease rating [a]
Stem	0	100	3.6
	5	98	3.0
	8	98	2.8
	9	85	2.3
	10	71	2.2
Leaf	0	100	2.0
	5	87	1.5
	8	88	1.8
	9	59	1.1
	10	23	0.6
Pod	0	100	2.8
	5	79	1.4
	7	73	1.2
	8	53	0.6
	9	3	0.2
Seed	0	69	2.7
	3	66	2.4
Controls		2	0.1

[a] Based on a scale of 0–5 (0 = no infection; 5 = all plants dead or dying).

TABLE 2. Development of bacterial wilt in Grimm alfalfa inoculated in the greenhouse with stems and leaves from 5 samples of infected alfalfa hay stored dry at room temperature for 10 years

Hay sample	Avg. disease rating[a] produced by	
	Stems	Leaves
1	1.8	0.5
2	2.9	0.6
3	2.0	0.2
4	2.5	1.4
5	1.7	0.4
Control	0.1	0.1

[a] Based on a scale of 0–5 (0 = no infection; 5 = all plants dead or dying). LSD at 1% level, 0.6.

The infective capacity of the pod debris was similar to that of the leaves (Table 1), except that it declined more rapidly after 5 years of storage. After 9 years, the remainder of the material produced very little infection.

Few tests were made on infected alfalfa seeds since they were very scarce and difficult to obtain (3). In two samples studied (Table 1), the wilt organism was still highly infective after 3 years of storage.

Very little disease occurred in the pots of control plants scattered throughout each experiment (Table 1). Occasional infection was attributed to spread of

the organism from adjacent pots of plants inoculated with the test materials.

These results show that the bacterial wilt organism can remain viable and virulent for long periods in dry portions of infected plants. Alfalfa hay, seed, and pod debris in poorly cleaned seed may thus harbor the organism, even after storage for several years, and provide a source of infection when used locally or in other areas.

LITERATURE CITED

1. Ark, P. A. 1958. Longevity of Xanthomonas malvacearum in dried cotton plants. Plant Disease Reptr. 42:1293.
2. Chamberlain, D. W. 1957. Maintaining bacterial organisms in soybean leaves. Plant Disease Reptr. 41: 1039-1040.
3. Cormack, M. W., and J. E. Moffatt. 1956. Occurrence of the bacterial wilt organism in alfalfa seed. Phytopathology 46:407-409.
4. Cormack, M. W., R. W. Peake, and R. K. Downey. 1957. Studies on methods and materials for testing alfalfa for resistance to bacterial wilt. Can. J. Plant Sci. 37:1-11.
5. Jones, F. R. 1928. Development of the bacteria causing wilt in the alfalfa plant as influenced by growth and winter injury. J. Agr. Research 37:545-569.
6. Kernkamp, M. F., and G. Hemerick. 1952. A "deep-freeze" method of maintaining virulent inoculum of the alfalfa wilt bacterium, Corynebacterium insidiosum. (Abstr.) Phytopathology 42:13.

A COMPARISON OF ASCOSPORE CARRIERS FOR INOCULATING ALFALFA WITH PSEUDOPLEA TRIFOLII

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Selection for resistance in alfalfa (*Medicago sativa* L.) and clovers (*Trifolium* spp.) to the leaf spot caused by *Pseudoplea trifolii* (Rostr.) Petr. has lacked a suitable method for producing sufficient inoculum to screen a large number of plants. This fungus does not produce conidia and, since previous work has showed that comminuted agar cultures are noninfectious, ascospores must be used to inoculate plants. A method, using ascospores ejected from sporulating V-8 juice agar cultures in petri dishes inverted over plants, has been employed to obtain abundant infection but it is not practical for large scale inoculations (2). To find a carrier for the quantity of ascospores required for such inoculations, a comparison was made of several liquids and dusts as to their suitability for collecting and storing ascospores for later application to plants.

Ascospores were produced in quantity on V-8 juice agar in petri dishes inoculated by inverting them over sporulating cultures. The uniform inoculation of the whole plate by this method resulted in a dense production of perithecia which began discharging ascospores in 5 to 10 days depending on the isolate employed. Ascospores were collected by placing inverted sporulating cultures on petri dish bottoms containing 10 ml of the carrier. The carrier containing ejected ascospores was decanted into a flask every 2 days and a fresh supply of it was replaced under the same culture. A culture usually continued to produce ascospores over a 10-day period. One petri plate of a heavily sporulating isolate generally produced sufficient ascospores to make 50 ml of a suspension containing approximately 30,000 ascospores per ml.

Talc, Bayol D (a product of Esso Standard Oil Co.), ethylene glycol, and glycerol were tested for the following desirable characteristics: (1) inhibition of ascospore germination during the collection period, (2) noninhibition of ascospore germination and infectivity when applied to plants, (3) nonphytotoxicity to inoculated plants, and (4) suitability as a spore storage medium. Talc, and the nonphytotoxic, low viscosity mineral oils of which Bayol D is a representative, have been used as carriers for rust urediospores (3). Ethylene glycol and glycerol have been used as suspending media for sperm cells and spores of microorganisms (1).

Talc met all of the requirements of a good carrier except one—very few leaf spots developed on inoculated alfalfa. Only slight infection was obtained when the mixture of talc and ascospores was dusted on dry leaves or on leaves which had been sprayed with water or a 0.25% gelatin solution. Numerous colonies developed on agar plates dusted with the mixture when inoculations were made. Decolorized, inoculated leaves were examined; a majority of the ascospores had germinated and formed a mycelium which grew over the surface of the leaf and did not penetrate. The ascospores remained viable in the talc for 2 years at room temperature.

No ascospore germination occurred in Bayol D during the collection period and no inhibition of germination occurred when the ascospores were sprayed on plants or agar plates. Ascospores remained viable in the oil suspension for 9 months at room temperature. Bayol D was slightly phytotoxic; a deterioration of the roots of 1-month-old seedlings occurred about 2 weeks after inoculation. This disadvantage could be minimized by transplanting selected seedlings into fresh soil immediately after disease readings were made. No such deterioration of the roots of older plants occurred, and no other symptoms of phytotoxicity were observed on other parts of the plants.

Ethylene glycol solutions of 10 and 25% were toxic to ascospores of *P. trifolii*. Percentage germination of the ascospores in these 2 solutions when sprayed on agar plates was only 8 and 2% respectively, so this compound was not tested further.

Glycerol solutions of 10, 20, 25, 30, 40, 50, and 100% were tested. Most of the ascospores germinated in the 10 and 20% solutions during the collection period; a few germinated slowly in the 25% solution; no germination occurred in the remainder of the solutions. Later collections were made only in solutions containing more than 25% glycerol which inhibited spore germination completely. When sprayed on agar,

all ascospores in each solution germinated. Fairly good infection developed on seedlings and older plants when inoculated with ascospores suspended in glycerol solutions plus 0.25% gelatin. Ascospore viability decreased after 2 weeks in the glycerol solutions at room temperature and when frozen.

Ascospores collected in oil or glycerol have been used in the greenhouse to inoculate flats of seedlings of different alfalfa varieties with satisfactory results. Both carriers offer promise as methods of testing large populations of alfalfa and clovers for resistance to Pseudoplea leaf spot.

LITERATURE CITED

1. Fennell, Dorothy I. 1960. Conservation of fungous cultures. Botan. Rev. 26:79-141.
2. McDonald, W. C. 1958. The Pseudoplea-Stemphylium complex on alfalfa. Phytopathology 48:365-369.
3. Rowell, J. B., and E. B. Hayden. 1956. Mineral oils as carriers of urediospores of the stem rust fungus for inoculating field-grown wheat. Phytopathology 46:267-268.

ADDITIONAL INDICATOR PLANTS FOR EXOCORTIS AND EVIDENCE FOR STRAIN DIFFERENCES IN THE VIRUS

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The virus nature of exocortis was first established in 1950 (1) through its transmission to *Poncirus trifoliata* (L.) Raf. Since then various mandarin-limes (*Citrus limonia* Osbeck), and citranges (*P. trifoliata* × *C. sinensis* (L.) Osbeck) have been reported to show symptoms of exocortis (2, 5, 6). Recent studies (7) have indicated that exocortis virus also induces bark-splitting in varieties of sweet lime, *C. limettioides* Tanaka.

This paper reports sweet lime, sweet lemon (*C. limetta* Risso), and Cuban shaddock (a lemon hybrid), as indicators of exocortis virus. Some evidence for strains of exocortis virus differing in incubation periods in susceptible hosts and reaction to phloroglucinol-HCl in *P. trifoliata* also is presented.

In 1953, a routine screening of various citrus species and varieties was begun in search of a rapid indicator plant for exocortis virus. Exocortis-infected budwood used in this program came from different old-line Eureka lemon strains that had induced characteristic exocortis symptoms in either *P. trifoliata*, Morton citrange, or mandarin-limes. Later tests showed that the source trees used were free of other known citrus viruses. Seedlings of test varieties were selected for uniformity and graft-inoculated with scions from the exocortis source trees. In most cases scions were grown to form the top of the tree; in some plants the scion growth was suppressed. Trees grafted with scions from exocortis-free lemon trees served as controls.

Of 84 citrus species and varieties, in addition to *P. trifoliata* and its hybrids and mandarin-limes, tested thus far, only Palestine sweet lime, Cuban shaddock, and Dorshapo sweet lemon show symptoms attributable to exocortis infection (Fig. 1). Symptom development in these hosts usually follows the same pattern. The first external symptom consists of shallow narrow splits resembling growth cracks expanding to an inch or more in width, in the bark of the trunk. In sweet lime the cracks may extend almost into the wood. New bark forming below the cracks fills them as the trunk expands. In Cuban shaddock, bark scaling similar to that in mandarin-limes develops usually below the soil level, following the bark-splitting. Symptoms in sweet lemon were much less severe than in sweet lime and Cuban shaddock. Affected trees were generally quite stunted. Dwarfing was more severe in Cuban shaddock than in sweet lemon or sweet lime. No leaf, bark, or wood symptoms of any kind developed in any of the other 81 graft-inoculated plants or the control plants.

Some variation in the onset of the symptoms of exocortis was noted among plants of single varieties inoculated from different sources and among plants of different varieties inoculated from a single source. To confirm these observations, a comparative study was made of the variation in the incubation period of 3 different indicator rootstocks (Cuban shaddock, Palestine sweet lime, and *P. trifoliata*) when grafted with scions from 3 different sources of exocortis virus (CES, Cascade, and Hughes Eureka lemon).

Palestine sweet lime and *P. trifoliata* stocks grafted with scions either from Cascade or Hughes Eureka developed initial exocortis symptoms 2½-3½ years after grafting; those grafted with scions from CES Eureka developed symptoms 1½-2 years after grafting. Cuban shaddock stocks grafted with scions from Cascade or Hughes Eureka developed initial symptoms 3½-4½ years after grafting; those grafted with scions from CES Eureka developed symptoms 2½-3 years after grafting. These results show that the incubation period of any 1 source of exocortis virus in Palestine sweet lime is comparable to that in *P. trifoliata*. The incubation period of exocortis virus in Cuban shaddock appears to be longer than for sweet lime and *P. trifoliata*. These results further indicate that exocortis virus in CES Eureka has a shorter incubation period in all 3 indicator plants than the virus from the other 2 sources. The authors have also noted similar differences in reaction by Morton citrange to different sources of exocortis virus (3).

Bark samples were removed from the *P. trifoliata* rootstocks following the development of bark scaling and treated with phloroglucinol-HCl (4) to determine if this stock inoculated with different exocortis sources responded differently to this staining procedure. All stocks grafted with CES Eureka scions gave a stronger and more discernible color reaction than stocks grafted with the other scions.

Results of these studies show that the exocortis virus from CES Eureka behaves in a different manner than the exocortis virus from the other sources. In light

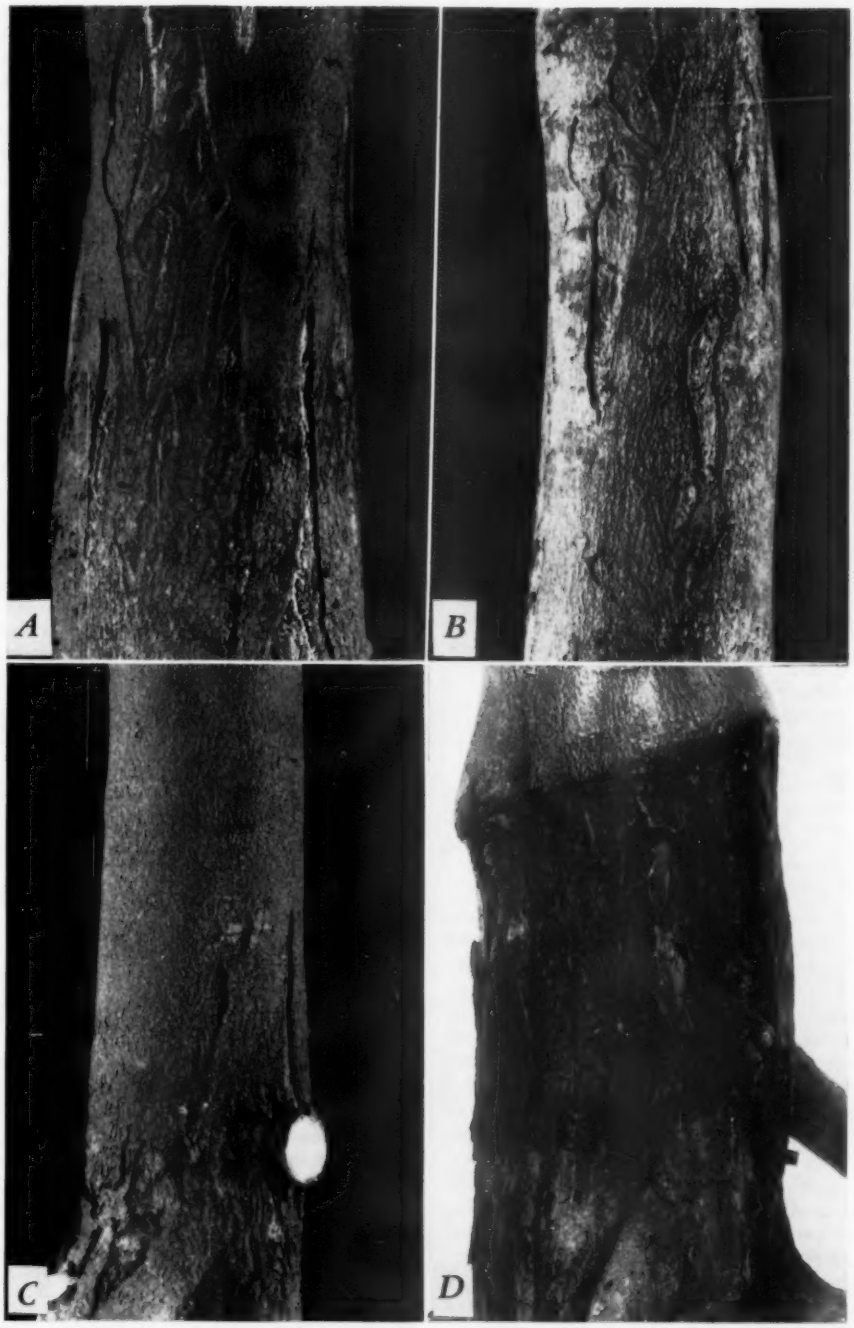

Fig. 1. Reactions of 4 citrus varieties to infection with exocortis virus. **A)** Bark-splitting in Palestine sweet lime. **B)** Bark-splitting in Dorshapo sweet lemon. **C)** Bark-splitting and scaling in Cuban shaddock. **D)** Bark-scaling in *Poncirus trifoliata*.

of the absence of other known viruses in the source trees, this suggests that the virus in CES Eureka is another strain.

LITERATURE CITED

1. Benton, R. J., F. T. Bowman, Lillian Fraser, and R. G. Kebby. 1950. Stunting and scaly butt of citrus associated with Poncirus trifoliata rootstock. Agr. Gaz. N. S. Wales 61:20-22, 40.
2. Bitters, W. P., J. A. Brusca, and N. W. Dukeshire. 1954. Effect of lemon budwood selection in transmission of exocortis. Citrus Leaves 34:1, 8-9, 34. (See also California Citrograph 39:70-71, 84-85.)
3. Calavan, E. C., and L. G. Weathers. Evidence for strain differences and stunting with exocortis virus. *In* W. C. Price, ed. Proc. 2nd Conf. Intern. Org. Citrus Virologists, in press.
4. Childs, J. F. L., G. G. Norman, and J. L. Eichhorn. 1958. A color test for exocortis infection in Poncirus trifoliata. Phytopathology 48:426-432.
5. Morfira, S. 1955. A moléstia "exocortis" e o cavolo de limoeiro cravo. Rev. Agr. (Piracicaba) 30: 99-112.
6. Olson, E. O., and A. V. Shull. 1956. Exocortis and xyloporosis—bud-transmission virus diseases of Rangpur and other mandarin-lime rootstocks. Plant Disease Reptr. 40:939-946.
7. Weathers, L. G., and E. C. Calavan. 1959. The occurrence of cachexia and xyloporosis in California lemon varieties, with particular reference to the old-lime Eureka lemon. Plant Disease Reptr. 43:528-533.

MUTABILITY OF PHYTOPHTHORA INFESTANS ON BLIGHT-RESISTANT SELECTIONS OF POTATO AND TOMATO

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Changes in the host range of *Phytophthora infestans* (Mont.) de Bary after continued culture on resistant varieties of potato have been reported by several investigators (2, 3, 5, 6). The phenomenon has been variously referred to as "educability," "building-up of virulence," and "adaptive parasitism." Shifts from specialization on potato to virulence on tomato have been observed (1, 4) as well as changes from race 0 to races 1.2.4, 1.4, and 2.4 after serial passages through the senescent leaves of the appropriate differential hosts (5).

In the course of testing large numbers of potato and tomato seedlings for blight resistance, we have encountered generally a susceptible condition in the juvenile and senescent phases of growth of resistant plants during which the fungus can sporulate to a limited degree. In the test material which consisted of various R genotypes, these 2 weak points were exploited to introduce races of the pathogen into otherwise resistant hosts and to allow its multiplication in their foliage.

The juvenile phase of susceptibility in resistant selections could be prolonged by the production of "giant" succulent leaves, induced by the decapitation of plants and by the removal of their lateral buds. The fungus also was cultured on yellowing senescent leaves. Inoculum for each race originated from single-zoospore cultures and was produced on detached leaves of Stokesdale tomato or Green Mountain potato in covered trays in a moist chamber at 15°C. Five days after the leaves were inoculated, sporangia were washed off into 100 ml of chilled tap water and the test trays of senescent or giant leaves of the resistant varieties were inoculated immediately. After 10-15 days on these substrates, any spores produced were transferred in tap water to leaves of the susceptible variety for a secondary build-up. A parallel series of passages through the susceptible hosts only was performed with each culture and race determinations were made at the time of each transfer.

The series of alternate passages through the resistant and susceptible varieties usually resulted in the appearance of a culture capable of attacking the resistant variety, although an equal or a greater number of generations on the universal suscepts yielded only the original race. Table I shows that the number of passages required to produce a shift in host range varied with the original race and with the resistant host. Races 1.2.3.4 and 1.2.4.5 were produced from race 1.2.4 after 3 passages through $R_1R_2R_3R_4$ and 4 passages through our seedling 3053-18, presumed to carry a gene R_5. Race 1.2.3.4 yielded 1.2.3.4.T_1 after 7 generations on New Hampshire Cherry Tomato, bearing the gene PhT_1 (7), and race 1.2.3.4.6 after 8 generations on a seedling with a possible gene R_6. A culture of Race 0 that shifted to race T_1 after 7 passages through PhT_1 remained unchanged, however, after 7, 4, and 12 passages, respectively, through hosts bearing the R_1, R_2, and R_3 genes.

In breeding potatoes for blight resistance, the valuable partial or field resistance is nonspecific with respect to races of the pathogen and is controlled by unelucidated genetic factors. In seedling populations of *Solanum demissum* × *S. tuberosum* origin, it is masked by the presence of the R genes that control the hypersensitive type of resistance to individual races. A realistic and idealistic approach to this problem of masked partial resistance would be to push the fungus to the limits of its ability to attack the resistant species. The full complement of genes controlling the hypersensitive reaction in a given species would then be known and the race capable of attacking plants bearing all the genes for resistance in the species could be used to select breeding material with partial resistance.

Race 1.2.3.4.6 was put to practical use to screen a large family of *S. demissum* × *S. tuberosum* derivatives for partial resistance to blight. This could not be done with race 1.2.3.4, because of the presence of genes conferring hypersensitivity to it and lower races in a large proportion of the seedlings. The details of these tests will be reported elsewhere.

Such a ready response to manipulation of the fungus naturally invites speculation. On the basis of our

TABLE 1. Selection of races of *Phytophthora infestans* (Mont.) de Bary induced by serial passage through foliage of resistant hosts

Original race	Genotype of host	Condition of foliage	No. of passages	Final race
0	Ph_1	senescent	7	T_1
1.2.4	$R_1R_2R_3R_4$	senescent	3	1.2.3.4
1.3.4	$R_1R_2R_3R_4$	senescent	6	1.2.3.4
1.2.4	R_3 (?)	senescent	4	1.2.4.5
1.3.4	R_3 (?)	senescent	7	1.3.4.5
1.2.3	R_5 (?)	senescent	5	1.2.3.5
1.2.3.4	Ph_1	senescent	7	1.2.3.4.T_1
1.2.3.4	R_6 (?)	giant-juvenile	8	1.2.3.4.6

observations, we exclude oospores as a variability mechanism in the field in our northern environment. If the fungus is permitted entry to the resistant host in either the juvenile or the senescent phase of its development, it seems logical that it will grow and mutate. A few mutant nuclei in the mycelium may carry a factor for pathogenicity towards the resistant host. The physiological state of juvenile or senescent leaves that allows a saprophytic development of the fungus, plus the selective effect of any "residual" resistance upon the heterocaryon created by mutation, probably permits the survival of the appropriate race. Mutation of the original race to a wider host range may take place in one step, but in serial host passages it may be the result of a series of step-mutations.

LITERATURE CITED

1. DE BRUYN, HELENA L. G. 1947. Het rassenproblem bij Phytophthora infestans. Vakbl. Biol. 10:147-152. (Abstr.) Rev. Appl. Mycol. 29:379.
2. DE BRUYN, HELENA L. G. 1951. Pathogenic differentiation in Phytophthora infestans (Mont.) de Bary. Phytopathol. Z. 18:339-359.
3. MILLS, W. R. 1940. Adaptive parasitism of Phytophthora infestans. (Abstr.) Phytopathology 30:17.
4. MILLS, W. R. 1940. Phytophthora infestans on tomato. Phytopathology 30:830-839.
5. MILLS, W. R., and L. C. PETERSON. 1952. The development of races of Phytophthora infestans (Mont.) de Bary on potato hybrids. (Abstr.) Phytopathology 42:26.
6. REDDICK, D., and WILFORD MILLS. 1938. Building up virulence in Phytophthora infestans. Am. Potato J. 15:29-34.
7. Report of the Tomato Genetics Cooperative. 1960. 10: 3.

LETTERS TO THE EDITOR

A POSSIBLE HISTORY OF SOUR CHERRY YELLOWS

Certain virus diseases of stone fruits have been known for a considerable time—peach yellows can be traced back reliably to 1791. Others seem recent in origin: they may have remained unrecognized, they may have mutated from a pre-existing virus of little importance, they may have been introduced into new host plants, or they may have arisen *de novo*.

The history of sour cherry yellows, a widespread and serious disease of cherry, is well documented from the time its virus etiology was established in 1939. Is this virus of recent origin in cherry? Or is the virus much older and was the disease merely unrecognized?

The cardinal symptoms of sour cherry yellows in the cherry varieties Montmorency and Early Richmond have been summarized by Keitt *et al.* (5) as: a striking green and yellow leaf mottle that appears, under appropriate temperature conditions, about 3 or 4 weeks after petal-fall; a much reduced spur system; and a reduction in fruit yields up to 50% or more. Diseased trees eventually develop a willowy growth habit with long bare spaces on the twigs. The fruits produced are larger than normal and of excellent quality (5).

Although the virus etiology of sour cherry yellows was first demonstrated by Keitt and Clayton (6) in 1939, the initial description of the characteristic foliage mottle seems to have been made by Stewart (11) in 1919. He considered "yellow-leaf" of sour cherry a non-parasitic disease caused by "unfavorable soil and weather condition." Gloyer and Glasgow (2) reported that "physiological yellow-leaf" of Montmorency developed in trees "lacking vigor and growth" or in trees "suffering from winter injury of the roots." Their observation of the connection of winter injury and sour cherry yellows was valid; although, in the light of present knowledge, cause and effect were reversed. There is little question that the "physiological yellow-leaf" of Stewart and Gloyer and Glasgow was sour cherry yellows, and these 2 accounts have been generally accepted as the initial descriptions of this disease (5).

The reduction in fruit yields, increase in fruit size, and willowy growth habit that accompany sour cherry yellows have long been known. Two variants of the variety Montmorency were distinguished by Duhamel (1) as early as 1768. He described "Large-fruited Montmorency (Gros Gobet, Short-stemmed Montmorency)" and commented: "It sets its fruit with difficulty and ordinarily yields not much—so that it is sometimes called 'The Fruit-aborter' . . . It is so little abundant that its culture is neglected; it is found only as a curiosity and in gardens not devoted to utility alone." His description of Montmorency Ordinaire or Long-stemmed Montmorency is essentially identical in tree and leaf characters to that of Gros Gobet, but in his description of the fruit, he stated: "The fruit are (*sic*) smaller than Gros Gobet . . . This cherry ripens in early July before Gros Gobet. Moreover, it is a little inferior in size and goodness; however, it is increasing, and is the cherry grown in preference to Gros Gobet because it is much less subject to fruit abortion and produces more fruit."

Prince (9) in 1832 and Mortillet (8) in 1866 also remarked on the scanty yields of Gros Gobet. Mortillet (8) placed Gros Gobet and Montmorency de Bourgueil in a "less meritorious" class; he described the fruit of Gros Gobet as "medium to large . . . the tree very infertile," while of Montmorency de Bourgueil, he commented: "This cherry is very similar to Gros Gobet and seems to me not very fertile." Trees of Montmorency Ordinaire, on the other hand, were "very fertile" (8).

Hedrick (3), in his definitive work on American cherry varieties, described anew the variants of Montmorency: Large Montmorency, Montmorency, and Bourgueil. In relating the history of these variants (p. 154), he stated: "At any rate, there have come to be at least three distinct types of Montmorency: the true Montmorency with long stems and moderate sized fruit . . .; the Large Montmorency with its large fruit and shorter, thicker stems . . .; and the Short-stem Montmorency . . . In 1875, Ellwanger and Barry, Rochester, New York, disseminated this sort (Large Montmorency) quite extensively, but it proved too unproductive for commercial use. . . The unproductiveness of this cherry has been consistently mentioned by nearly every writer from Duhamel's time to the present."

As in the case of Montmorency, early pomological litera-

ture mentioned 2 variants of the variety Kentish, better known in the United States as Early Richmond. M'Intosh (7) was apparently the first to point out (1839) differences in the varieties Kentish and Flemish, which he considered synonymous: "Kentish (synonym—Flemish)—supposed to have been introduced from Flanders by Harris, fruiterer to Henry VIII, and first planted in Kent near Sittingbourne, which accounts for both names . . . their difference consist (*sic*) in the one being a great and the other a moderate bearer, and that one has drooping shoots, while the other's are more upright—circumstances which we expect arise from cultivation, soil, or the constitutional vigour of the trees, but certainly not sufficient to stamp them distinct kinds." Hogg (4), however, maintained that Kentish and Flemish were distinct varieties. Of Flemish, he said: "Pomologists have fallen into great mistakes with this cherry, particularly those who think it synonymous with Gros Gobet; others think it the same as Kentish. The latter is nearer the truth, but the Kentish and the Flemish are decidedly different. The fruit of the two could not be distinguished the one from the other; but the trees of the Flemish are less drooping than those of the Kentish and the fruit is smaller and about eight or ten days later." Hogg's observations were repeated essentially *verbatim* in 1873 (10).

Thus, 2-3 variants of Montmorency have been consistently distinguished throughout the 200-year history of this variety since Duhamel's time. These variants were singled out by differences in fruit size, fruit quality, pedicel length, and tree fertility, the very differences induced by sour cherry yellows. With the variety Early Richmond, the history of variants is less certain and less detailed, but it seems clear that Kentish and Flemish differed largely in tree fertility and drooping vs. upright growth habit—characteristics again likely to be altered by sour cherry yellows.

None of these accounts mentions the occurrence of foliage mottle and defoliation, symptoms which would afford conclusive evidence of the presence of sour cherry yellows. These distinctive symptoms are, however, of rare occurrence in the warmer areas of cherry cultivation, and very similar foliage symptoms are induced by certain fungus and bacterial diseases of cherry. While it is possible that the observations recounted here were actually true descriptions of cherry varieties, some of which are now lost or confused, it seems equally possible that they are actual records of the effects of sour cherry yellows on the varieties Montmorency and Early Richmond. If the latter possibility is true, then sour cherry yellows is of French origin and has been present in sour cherries for at least 200 years. The causal virus was then introduced into the United States with Large Montmorency, a variety that "probably came to America . . . early in the Nineteenth Century" (3).

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LITERATURE CITED

1. Duhamel du Monceau, M. 1768. Traite des Arbres Fruitiers 1:180-182. Paris.
2. Gloyer, W. O., and H. Glascow. 1928. Defoliation of cherry trees in relation to winter injury. N. Y. (Geneva) Agr. Expt. Sta. Bull. 555.
3. Hedrick, U. P. 1915. The cherries of New York. II. N. Y. (Geneva) Agr. Expt. Sta. Rept. for 1914.
4. Hogg, Robert. 1860. The Fruit Manual. Cottage Gardener Office, London.
5. Keitt, G. W., G. H. Berkeley, Donald Cation, C. N. Clayton, E. M. Hildebrand, J. D. Moore, and E. J. Rasmussen. 1951. Sour cherry yellows. *In* Virus diseases and other disorders with viruslike symptoms of stone fruits in North America. U. S. Dept. Agr. Handbook 10:152-158.
6. Keitt, G. W., and C. N. Clayton. 1939. A destructive bud-transmissible disease of sour cherry in Wisconsin. Phytopathology 29:821-822.
7. M'Intosh, Charles. 1839. The Orchard. W. S. Orr & Co., London.
8. Mortillet, M. P. de. 1866. Les meilleurs fruits: Le cerisier 2:204-205. Prudhomme, Girond & Cie., Grenoble, France.
9. Prince, William. 1832. The Pomological Manual. New York.
10. Scott, J. 1873. Scott's Orchardist or Catalogue of Fruits. 2nd Ed. H. M. Pollett, London.
11. Stewart, F. C. 1919. Notes on New York plant diseases. II. N. Y. (Geneva) Agr. Expt. Sta. Bull. 463.

ON "FACULTATIVE HETEROECISM"

Recent use of "facultative heteroecism" in a new sense (3) suggests the need for a clarifying note on this 50-year-old, but still undefined, term. "Facultative heteroecism" has denoted several fascinating phenomena that deserve study—but preferably with a distinct name for each. The term has been used to denote life cycles of the following groups.

1) Rusts that are "more or less independent of the supposed alternate stage . . ." (6). This would cover a great many species that spread chiefly or exclusively by means of urediniospores, but also it includes perennial species and those having repeating aeciospores (a special group, considered below). "Sporophyte-repeating" would be a meaningful term for the rusts spreading by urediniospores.

2) Heteroecious rusts that "can be transmitted directly from pine to pine by infection with aeciospores . . ." (6, 7; commonly used in forest pathology). If aecial hosts other than pine are included, there are about 12 supposedly heteroecious species to which repeating aeciospores have been ascribed, 6 of them in *Cronartium*. It has not been confirmed, in spite of numerous attempts, that spores from a given aecium are capable of infecting both the sporophytic and gametophytic hosts; it is more likely, for most of the species, that separate races follow the long and short cycles. Therefore, one may doubt whether there is anything "facultative" about the cycles. In the short cycled races, aeciospores (in most instances, binucleate) give rise to haploid mycelia without forming basidia; cytological details are unknown. Rusts whose aeciospores give rise to haploid mycelia could be called "gametophyte-repeating."

3) Rusts that are "capable of producing both generations on the host of one generation" (4). This concept harks back to Fischer's hypothesis (2) of the origin of heteroecism: that ancestral species were plurivorous, and able to live their whole life cycles on either of the 2 present hosts. Fromme (4), who thus limited use of "facultative heteroecism," did not suggest that such fungi exist, and indeed no examples are known of whole life cycles on both hosts. The sporophyte of *Puccinia graminis*, however, can occur on barberry (1) and its pycnia have been produced on wheat (5); there is no clear name for this surprising versatility in life cycle. "Plurivorous" is still a good term for a rust that parasitizes many different hosts.

4) A rust (*Melampsora lini*) that is an autoecious euform but cannot develop both generations on certain host varieties (3). Thus there can be an alternation between two sets of hosts. "Heteroecism," with its long established connotations, seems unsuitable here. Nor does it seem likely that this sort of host alternation can be an evolutionary stepping stone to heteroecism in the usual sense.

It is hoped that listing of the several meanings employed (all in PHYTOPATHOLOGY) will discourage further use of the ambiguous term, "facultative heteroecism." The term should apply to the life cycle of an organism able in 1 spore stage to "repeat" on its host and to alternate to an unrelated host. Meinecke's report (7) seems unconfirmable; no organism fitting this definition is known.

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LITERATURE CITED

1. CRITOPOULOS, P. F. 1947. Production of teliospores and uredospores of Puccinia graminis on Berberis cretica in nature. Mycologia 39:145-151.
2. FISCHER, E. 1898. Entwicklungsgeschichtliche Untersuchungen über Rostpilze. Beitr. z. Kryptogamenflora d. Schweiz 1:1-121.
3. FLOR, H. H. 1959. Differential host range of the monocaryon and the dicaryons of a eu-autoecious rust. Phytopathology 49:794-795.
4. FROMME, F. D. 1916. Facultative heteroecism (?) of Peridermium harknessii and Cronartium quercus. Phytopathology 6:411-412.
5. JOHNSON, T., and G. J. GREEN. 1954. The production by Puccinia graminis of abortive pycnia on wheat. Can. J. Agr. Sci. 34:313-315.
6. MEINECKE, E. P. 1916. Peridermium harknessii and Cronartium quercuum. Phytopathology 6:225-240.
7. MEINECKE, E. P. 1920. Facultative heteroecism in Peridermium cerebrum and Peridermium harknessii. Phytopathology 10:279-297.

THE PRESIDENT'S COLUMN

The Analyses of Membership initiated last month is concluded with the "Fields of Interest." There is relatively little change among the 6 sections of Table 3, except that only 18% of the members are interested in Control Measures, compared with 22% in 1953. Within Crop Diseases, interest in tropical crops has increased from 3 to 5%. This is reflected in the demand for a Caribbean Division. Vegetable diseases have replaced Fungicides as the greatest single source of interest. The outstanding relative increase, however, is for Nematology which in section B changed from 9 to 32%. This is in accord with the growth in the profession of nematology. Surprisingly, interest in Fungus Physiology has fallen from 27 to 13%.

A third Directory is due in 1963. It is hoped that it then will be possible to prepare an index of the Fields of Interest which should appreciably increase the usefulness of the Directory.

S. E. A. McC.

TABLE 3. Fields of interest reported by number of members indicated

A—Diseases of Crops			
Vegetable (incl. tomato)	237	Citrus	30
Cereal, small grain	173	Cotton; tobacco—each	28
Forest, shade tree	142	Forest products, wood decay	20
Fruit	135	Market, transportation, storage	20
Ornamental	106	Corn; sugarcane—each	15
Forage	79	Bean	14
Potato	59	Banana; soybean—each	13
Tropical, subtropical	55	Grape; nursery crop—each	12
Root	50	Turf	11
Field	45	Sugar beet	10
Small fruit	33	Miscellaneous	38
B—Microorganisms			
Nematology	128	Fungus taxonomy, morphology, cytology	15
Mycology (incl. special groups)	113	Soil fungi	14
Fungus physiology	53	Smuts and rust	12
Fungus genetics	36	Bacteriology	7
Microbiology, soil microbiology	26		
C—Viruses			
Virology	181	Stone fruit viruses	23
Virus diseases	47	Insect vectors	14
D—Other plant pathology			
General plant pathology	124	Plant disease survey	20
Teaching	35	Soil-borne plant pathogens	20
Physiology of parasitism	34	Gas injury, air pollution	13
Bacterial diseases	27	Nature of disease resistance	11
Diseases: physiological, fungus, wilt, etc.	28	Plant pathological anatomy	10
Epidemiology and ecology of disease	25	Seed-borne diseases	10
Physiology and biochemistry of disease	20	Miscellaneous	28
E—Control measures			
Fungicides	224	Seed treatments	19
Breeding for disease resistance	101	Plant quarantine, regulatory work	15
Antibiotics	39	General plant disease control	15
Soil treatments	26	Fungicidal action; herbicides—each	14
Chemotherapy, systemic fungicides	24	Insecticides	10
Nematocides	23	Miscellaneous	38
F—Allied interests			
Breeding and culture: various crops	63	Plant physiology	10
Biological sciences: various	32	Industrial microbiology	9
Biochemical, biophysical sciences	15	Plant hormones; tissue culture—each	6
Entomology	11	Miscellaneous	27

NEWS

Editor: K. W. KREITLOW, Plant Industry Station, Beltsville, Md.

The Potomac Division held its 18th annual meeting at the Plant Industry Station, Beltsville, Md., March 2-3, 1961. Approximately 140 persons attended the presentation of 44 papers. Guest speaker for the evening banquet was Dr. H. A. Rodenhiser who gave an illustrated talk on his recent trip to Egypt. New officers elected were: President, J. G. Moseman; Vice-President, H. L. Barnett; Councilor and alternate, A. B. Groves and L. C. Cochran, respectively. The 1962 meeting will be held at West Virginia University.

An Index of Photographs in Phytopathology, Vols. 1-41 was prepared by G. K. Parris, Chairman, Botany Department, Mississippi State University. This unbound, mimeographed manuscript of 233 pages, known as Miscellaneous Publication No. 2 of the Botany Department, Mississippi State University, and limited to about 130 copies, has been deposited with plant pathological libraries and also placed in the hands of a number of teachers of plant pathology in the United States and abroad.

Professor L. J. Tyler, Department of Plant Pathology, Cornell University, returned recently to Ithaca after a sabbatical leave of 6 months. Prof. Tyler continued his work on diseases of small grain crops with plant pathologists in England, Scotland, Denmark, West Germany, Austria, and France. His travel was made possible by a Rockefeller Foundation grant.

Visiting biologist J. L. Harley, University Demonstrator in Plant Nutrition in the Department of Agriculture at the University of Oxford, was a visitor to the Plant Pathology Department at Cornell University in January. During his two weeks stay, he delivered a series of 6 lectures on "The Rhizosphere and Mycorrhizae" and consulted with staff and students on the physiology of the fungi.

Michigan State University began its Undergraduate Research Participation Program in 1959 with 19 students working in the Departments of Zoology, Entomology, Microbiology and Botany, and Plant Pathology. The program was continued for a second year under the supervision of the Division of Biological Sciences. Thirteen students worked on projects during the summer session and 14 projects are now under way.

Phytopathologist Alfredo Campos was elected president of the newly formed Mexican Phytopathological Society at the 2nd National Congress of Entomology and Plant Pathology held in Chapingo, Mexico. Dr. Campos is currently Head of Plant Pathology in the Colegio de Post-Graduados, National School of Agriculture, Chapingo, Mexico.

Virologist Karl Maramorosch, formerly associated with The Rockefeller Institute, has been appointed a senior staff member of The Boyce Thompson Institute for Plant Research, Yonkers 3, New York. In his new position, Dr. Maramorosch will continue his investigations with insect vectors of plant viruses. Dr. Maramorosch was associated with the late Dr. L. O. Kunkel during the last 12 years and he brought back to Yonkers not only part of the old tradition but also the oldest colony of Aster Leafhoppers—now in its 37th year.

Phytopathologist James B. Kendrick, Jr., University of California, Riverside, has been awarded a Senior Postdoctoral Fellowship by the National Science Foundation to support his research studies abroad on some quantitative aspects of the biology of root infections by fungi. Dr. Kendrick will be on sabbatical leave for one year beginning March 15, 1961. He will be associated with Dr. S. D. Garrett at the Botany School, Cambridge University, England, until October 14, 1961, and with Dr. E. W. Buxton in the Department of Plant Pathology, Rothamsted Experimental Station from October 15, 1961 to March 15, 1962.

Professor K. G. Parker, Department of Plant Pathology, Cornell University, was presented a citation on January 18 by the New York State Horticultural Society in appreciation of his more than 30 years of work with fruit growers in the State. The award was made at the Rochester meeting of the Society.

The Cotton Disease Council held its 21st annual meeting at Greenville, South Carolina, January 9-10, 1961. This was prior to and in conjunction with the Beltwide Cotton Production and Mechanization Conference, January 11-13, which is sponsored by the National Cotton Council. There was a good representation of cotton pathologists from across the Cottonbelt. A half-day session was devoted to papers regarding bacterial blight research, present status and problems. The second day was given to reports of the various committees, each regarding specific problems in cotton diseases and to the business session in which Dr. C. D. Ranney, Mississippi Delta Branch Experimental Station, was elected Council Chairman, and Dr. R. E. Hunter, Oklahoma State University, was elected Council Secretary.

Phytopathologist J. R. Bloom, Pennsylvania State University, University Park, Penna., is on leave from February 1, 1961 until July 1, 1961. Dr. Bloom will study and conduct research on nematodes and nematode diseases of plants during this period in the Department of Plant Pathology, North Carolina State College, Raleigh.

The 17th Tobacco Workers' Conference was held at Clemson College, Clemson, South Carolina, from January 16-18, 1961. Approximately 30 plant pathologists from 9 states attended the Tobacco Disease section of these meetings, wherein progress reports and latest developments in tobacco disease research were presented and discussed.

Virologist Francis O. Holmes has been granted leave of absence for one year from The Rockefeller Institute to undertake an assignment in the study of cadang-cadang disease of coconuts under the Food and Agriculture Organization of the United Nations. His headquarters will be the United Nations Technical Assistance Board, W. H. O. Building, Taft Avenue, at Isaac Pearl Street, Manila, Philippines.

The 14th Alabama Pest Control Conference was held February 21 and 22 in Birmingham, Alabama. Phytopathologist, U. L. Diener, President of the Alabama Association for the Control of Economic Pests, presided at the opening session. Topics discussed by plant pathologists of Auburn University included: "Diseases of ornamentals and their control," by R. L. Self; "Diseases of shade trees and their control," E. A. Curl; "Disease control of fruit and garden pests," U. L. Diener; "Cotton diseases," A. L. Smith; "Diseases of lawns and sods," R. T. Gudauskas; and "Pest control in Iran," Coyt Wilson.

The 13th International Symposium on Crop Protection will be held at the State Agricultural College of the University of Ghent (Belgium) on May 9, 1961. There will be sections on Phytopathology (including fungus, bacterial and virus diseases and their control), applied entomology (including nematology), crop protection, chemistry, toxicology of pest control chemicals. A feature this year is the organization of a special section of "Integrated chemical and biological pest control," for which 14 contributions from Western European countries were received. Papers presented at the Symposium will be published in full.

Deaths—Plant pathologist Syed Vaheeduddin died due to appendicitis on December 28, 1960. Dr. Vaheeduddin was a 1936 graduate of the University of Minnesota and at the time of his death was Headquarters Deputy Director of Agriculture (Research) at Rajendranagar, Hyderabad, India.